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PLANT PHYSIOLOGY

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FIFTY YEARS OF PLANT PHYSIOLOGY IN AUSTRIA[1]

L. LINSBAUER

Plant physiology in Austria took a powerful upturn after the year 1873, the year in which JULIUS WIESNER brought into existence the first (not only in Austria, but anywhere) plant physiological Institut. It was the period in which this science under the three great stars, WIESNER, SACHS, and PFEFFER made great and decisive strides forward. It is understandable that very soon a host of enthusiastic young men gathered around the great teacher; and so it is no wonder that there went forth from this school many men who in subsequent years occupied professional chairs in the Austrian universities. Among the plant physiologists are mentioned the following: A. BURGERSTEIN (Wien), J. CZAPEK (Prag), W. FIGDOR (Wien), V. GRAFE (Wien), G. HABERLANDT (Berlin), K. LINSBAUER (Graz), and H. MOLISCH (Wien).

The situation was changed by the World War. The Austria of today, as those from foreign lands know it, has now only the universities of Vienna, Graz, and Innsbruck, which will be considered in this paper.

It is characteristic of JULIUS WIESNER that he was no specialist; the field of research in which he worked was many sided. Notwithstanding, there were a few main problems which occupied him through many years: Chlorophyll formation; the relation of the plant to light, which led to his photometric investigations and to the development of his concept of *Lichtgenuss,* which was of importance in ecological research; the causes of orientation of plant organs (anisomorphy, exotrophy, anisophylly), to mention only a few.

WIESNER liked to put his experiences to the service of practical needs. Best known is his great work *"Die Rohstoffe des Pflanzenreiches";* less well known is the essential service which he rendered to the sugar industry when he introduced the dialysis procedure which has been used ever since.

There is a peculiar charm to be seen in the fact that WIESNER also had an interest in the more general scientific problems, and gave expression to his thoughts concerning them. In one of his most important and funda-

[1] Translated from the original German by Dr. Frank M. Andrews, Indiana University.

mental works, *Die Elementarstruktur und das Wachstum der lebenden Substanz,* he put forward the theory that the cells are not the ultimate units of living material, but are made up of a sum of plasomes which already possess all of the properties of life. At the same time he viewed the cell not as a mere aggregation of individual particles, and the life processes of the plant not as accidental occurrences; but he thought that the driving mechanism of the plant's life is regulated by an internal order which WIESNER called *Enharmonie.* Such trains of thought, which remind one of the modern conceptions of life as a fundamental unity, show a notable breadth of comprehension.

WIESNER visited the United States in a private capacity, so to speak, by way of a trip to the Yellowstone region where he undertook photometrical investigations. He also published a paper, *The development of plant physiology under the influence of the other sciences.* (Congress of Arts and Science. Universal Exposition, vol. V, pp. 103–124. St. Louis. 1904.)

HANS MOLISCH, the successor of WIESNER, occupied the entire last half century with his scientific investigations. It is utterly impossible in a limited space to do justice to the labors of this industrious Nestor among Austrian botanists. We shall therefore attempt to give only a very general summary of his works, the number of which runs to about 200. His plant physiological investigations (apart from the purely anatomical ones) have enriched almost every chapter of this science. Here there are mentioned only a few remarkable papers which, as independent publications of preliminary studies are united into a complete account: *Die Pflanze in ihren Beziehungen zum Eisen* (1891); *Untersuchungen über das Erfrieren der Pflanzen* (1897); *Studien über den Milchsaft und Schleimsaft der Pflanzen* (1901); *Leuchtende Pflanzen* (1904); *Die Purpurbakterien* (1907); *Die Eisenbakterien* (1910).

The microchemistry of plants has experienced extraordinary advancement through numerous individual investigations by MOLISCH, and his *Mikrochemie der Pflanzen* (1913) is indispensable to every plant physiologist and plant anatomist. It should be mentioned in this connection, that plant chemistry and microchemistry were cultivated with success in Austria; well known is the fundamental *Biochemie der Pflanzen* by CZAPEK. In addition V. GRAFE worked in Vienna, and G. KLEIN wrote his *Histochemie der Pflanzen,* while general microchemical methods had as their outstanding devotees, PREGL and EMICH, in Graz.

MOLISCH's *Grundriss einer Histochemie pflanzlicher Genussmittel* (1891) had already served decidedly practical ends, and so he, the son of an old horticultural family, had especially at heart the needs of horticulture. He served these needs in his valuable study, *Das Warmbad als Mittel zum Treiben der Pflanzen* (1909). His main effort in this direction, however,

produced the standard work *Pflanzen Physiologie als Theorie der Gärtnerei,* which, having appeared in 1916, has since reached its sixth edition.

For the purpose of diagnosis in various fields, the paper *Aschenbild und Pflanzenverwandtschaft* (1920) offers valuable materials. MOLISCH also wrote a *Physiologie der Pflanzen* (1917), an *Anatomie* (1920), a fascinating book, *Botanische Versuche ohne Apparate,* and in addition a series of others in which he shows himself to be a master at popular presentation. All of the works of MOLISCH are distinguished by great exactness of observation, so that they possess documentary value. His arrangement of experiments is often of classical simplicity. And often, from apparently insignificant observations, he obtained important new facts. Because of the unusual breadth of the research field treated by him, the possibility presents itself to extend his work at many points. "That, indeed, is the best that a scientific work can offer, to give living impulse to new investigations." (WIESNER).

MOLISCH made extensive foreign trips. His impressions and observations in India and Japan, where he was invited to the organization of a biological institute, were reported in three books of travel, 1926, 1927, 1930. On his return trip he briefly visited the United States where he became acquainted with the famous Boyce-Thompson Institute. MOLISCH, who has recently become 80 years old, is vice-president of the Academy of Science in Vienna, and was at the time of the celebration of his birthday honored and accorded distinction from many places.

After MOLISCH, followed G. KLEIN, who concerned himself chiefly with plant chemistry; but he remained in connection with the institute only a short time.

During the years 1930–1934 F. C. VON FABER was the director of the WIESNER Plant Physiological Institute. As a tropical botanist with rich experience, and as a pioneer of free land botany from Java, he organized in the vicinity of Vienna a small free land station. Work along his line will be continued in Vienna. Before his departure for Munich he had modernized the institute.

With K. HÖFLER another of MOLISCH's students assumed the teacher's chair. HÖFLER is first of all a representative of cell physiology, which he developed in connection with several young co-workers. Also other representatives of this flourishing Austrian work have gone forth from MOLISCH's school, as WEBER in Graz, and GICKELHORN in Prag.

HÖFLER first studied the osmotic values of the plant cell. By the use of his plasmometric method which permits one to express the degree of plasmolysis of the protoplasm numerically, he investigated the permeability of various substances, and the distribution of permeability in the different tissues of a plant. He was able to show, moreover, that it is possible to

characterize certain systematic units of the plant kingdom by definite physical properties of the plasma, in the same way as it is possible to do from morphological and anatomical characters, and has thus shown the way to a "comparative protoplasmatics."

Jointly with R. CHAMBERS (New York) he investigated the tonoplast of *Allium cepa* whose membranes are looked upon as extremely thin fluid films composed of materials not miscible with water.

To round out my account I will mention that as older physiologists of the Vienna school, A. BURGERSTEIN (transpiration monograph) and W. FIGDOR (monograph on anisophylly) were still active. Moreover, investigations in the field of plant physiology were carried on in the technical agricultural school in Vienna, where J. BÖHM especially had occupied himself with the problem of sap ascent. Furthermore, from the Biological Experiment Station there were produced physiological papers under the leadership of FIGDOR and L. VON PORTHEIM and their students.

To these is added here O. RICHTER, likewise one of MOLISCH's students. At an early time in the now foreign University of Brünn he has for a long time made himself known by his physiological investigations of nutrition. He concerned himself with the nutrition of the algae, grew the Diatomaceae in pure culture, found that they can assimilate organic substances and that magnesium is indispensable for them, studied the relation of magnesium to the plant, and investigated the nutritional physiology of cultivated grasses. He showed the influence of impure (laboratory) air on physiological processes and so came to the study of narcosis.

The University of Graz maintained its interest in bringing out new relations between anatomy and physiology which indeed were always related to one another. It was G. HABERLANDT who, stimulated by SCHWENDENER's views, turned himself successfully on a large scale to the development of physiological plant anatomy. His fundamental work on this subject (1884) is universally known, as is also his work on the physiological tissue systems of the plant. By carrying over the concepts of animal physiology to the elucidation of structure and function of plant tissues, he came to the discovery of plant sense organs, which serve in part for the perception of light, and in part for the reception of mechanical stimuli. The announcement of this generally interesting discovery, and likewise his book, *Eine botanische Tropenreise* (1893), made him well known far beyond the circle of his own colleagues. The work which he accomplished after his call to Berlin does not enter into the present discussion.

He was followed in the year 1911 by KARL LINSBAUER. It is noteworthy that again through his work there was developed an intimate connection between anatomy and physiology, to be sure in another sense than in HABERLANDT's work. The great work originated and edited by him, *Handbuch der Pflanzenanatomie* (since 1926), sought a deeper penetration of

histology with the results of cytological, physiological, and mechanico-developmental investigations. As a contribution to this work he personally contributed the chapter on *Epidermis*. In the forefront of his interests stands the physiology of irritability. In the movement of tendrils he established the fact that in this response a hitherto unrecognized significance is to be attributed to torsions. The movement of stomata is to him not so much a mechanical as an irritability movement. With the aid of modern recording methods he ascertained the rate of stimulus transmission in the primary petiole of *Mimosa pudica,* found that conduction of stimulus in this plant can take place in the vessels, and that the sieve tubes are not necessary for this function. A great part of LINSBAUER's efforts were devoted to the problem of developmental mechanics and general biology.

After the death of LINSBAUER, FRIEDL WEBER assumed his position. By chance it was ordained that again anatomical and physiological investigations were joined in his work in a still different way; physiological changes of the individual cells were brought into relation to certain changes in state of their protoplasm. The pursuit of this idea led to the founding of a "protoplasmatic anatomy." Through research in such a direction, "protoplasmatics" became in Austria the focus of attention.

In addition WEBER, in the journal *Protoplasma* which he founded, has created an international organ which serves the whole field of physical chemistry of the protoplast. In addition he shared in the editorial responsibility for the *Protoplasma Monographien.*

In a few words we will now mention the University of Innsbruck at which E. HEINRICHER was active for decades. His main subjects of research were *Viscum, Lathraea,* and green hemi-parasites whose germination and nutritional relations he cleared up in a series of investigations. SPERLICH is his successor.

In the exchange of scientific thought, Austria has always participated, but seldom, and only transitorily, have botanists from foreign lands come to the Austrian professional chairs; Austria has always contrived to meet its need of academic teachers and investigators from its own people. It is significant for the new march of progress of the home-land research that scientific life has not become set in the mold of tradition, but has developed as a living continuum through a half century in which the individuality of scholars insures the continuous flow of ideas. On the other hand, our land can point to the fact that it has given a group of important investigators to neighboring nations. Among these may be mentioned VON GUTTENBERG (Rostock), G. HABERLANDT (Berlin), BRUNO HUBER (Tharandt), O. RICHTER (Brünn), STRUGGER (Griefswald); and at the Jugoslavian University of Agram (now Zagreb), VALE VOUK is engaged.

KLOSTERNEUBERG
OESTERREICH

FOLIAR DIAGNOSIS: PRINCIPLES AND PRACTICE[1]

WALTER THOMAS
(WITH SEVEN FIGURES)

Introduction

As a result of the broader view now taken with respect to the problems of soil fertility it is recognized that the conditions required for maximum growth must be sought from the facts of plant physiology as well as from those of soil science. For satisfactory growth a particular soil must satisfy certain conditions with respect to temperature, and to nutrient, water, and air supply to roots, the task of the investigator being to discover the best means of bringing the factors that govern these conditions under control. For this purpose the traditional chemical methods both of plant and of soil analyses have definite limitations; some have been abandoned, others are still in use.

In recent years interest has been renewed in the possibilities of the chemical analysis of the plant as a means of studying the nutrient relationships between the crop and the soil with respect to (1) the physiological requirement of a particular species and (2) the rate of supply of nutrients (**10**), *i.e.*, the supplying power of the soil (**27**). It is well established that the composition of a particular plant species is profoundly influenced by the composition of the soil (**40**). Consequently, in order to determine the influence of the soil on the action of fertilizers by means of analysis of the plant it is necessary to experiment first with plants growing in a relatively homogeneous soil (*vide infra*) using systematically laid out plots differentially fertilized. When this is done, the next step is to ascertain the nature of the variation in the composition of plants of the same species and variety in similarly treated (duplicate) plots, the soils of pairs of which may show all types of variations.

There are many reasons, however, why the analysis of the entire plant is not the best means of approach toward the problem of control under field conditions. Apart altogether from the disadvantages of having to dig up and analyze whole plants, the results obtained by the gross analysis of a mass of heterogeneous organs possessing different functions are not a sufficiently sensitive comparative index in reflecting the responses of the plant to differences in its environment (soil and weather) (**41**). The classical investigations of PIERRE (**34**) pointed to the sensitivity of the leaves to changes in composition resulting from differences in environment. But as long as no care was taken to differentiate between leaves of different ages little progress was made. The presentation of the gross analysis of leaves of all ages of a plant species, frequently seen even in present day literature,

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is of no service in diagnosis because neither the relative nor absolute amounts of the mineral entities are the same in leaves of different ages. Moreover, samples taken only at one period can give no information as to the course or nature of the changes in nutrition with growth in relation to differences in the medium. The further development of the method of leaf analysis ("diagnostic foliaire") as a means of studying the course of the absorption of mineral elements under the influence of different growth factors, especially those of fertilizer additions, is largely due to the labors of LAGATU and MAUME.

Principles of the method of foliar diagnosis

Of the numerous methods employed to control the nutrition of plants under the conditions of practical agriculture only the method of foliar diagnosis has any serious claim to be based on physiological facts established by consistent experimental results obtained over a long period of years (**15, 16, 17, 18, 19, 20, 21, 22, 26**). The laws governing the facts are still obscure, although glimpses of their working have been revealed. Some of the facts and their implications have already been discussed in a series of papers by the writer (**38, 39, 40, 41**).

FACTS UPON WHICH THE METHOD IS BASED

The experimental facts to which the method of foliar diagnosis is linked may be briefly summarized:

1. Two morphologically homologous leaves of the same species and variety are the seat of identical physiological processes when the medium (soil and meteorological factors) of the two is identical and, conversely, are the seat of physiological processes that are different when the medium is different (**13, 18, 19, 21**).

2. The composition of leaves of the same physiological (metabolic) age of plants grown on the same homogenous medium (soil and climatic) and receiving different fertilizer treatments reflects these differences in the sense that whenever a fertilizer element (whether N, P, or K) is effective, as determined by the response of the plant to that element, that response is always associated with an increase of that element in the dry foliage (**18, 19, 37**). This is not, as might appear at first, a principle *a priori*, because neither the processes of migration of the elements into the leaf from the stem nor export from the leaf into the stem is under consideration in the method of foliar diagnosis (*vide infra*), but only the amount of the element present in the leaf at the moment of sampling.

3. The composition—as determined by periodic analysis—of leaves of the same physiological (metabolic) age taken from plants of the same species growing on the same homogenous medium (soil and climatic) but receiving different fertilizer treatments are related to their development (**9, 18, 42**).

4. The magnitude of the variations in the composition, with respect to the dominant nutritive elements, of leaves of the same physiological age from plants of the same species grown in a homogenous soil and subjected either (1) to differential fertilizer treatment under the same meteorological conditions, or (2) to the resultant of meteorological factors acting in each particular growing season, is relatively quite large and, therefore, easily determinable. This delicate sensitivity of the leaf is no doubt related to its rôle as the laboratory of synthesis of the plant (**41**).

An "ideal" soil non-existent

Reference has been made to a "homogenous" (uniform) soil. Such an ideal soil, of course, does not exist in nature. If an area of land is divided into two plots, A and B, which are managed and fertilized alike and are sown at the same time with selected uniform seed, the yields may be different even when the land appears uniform to the eye. The yields may and, generally, will be widely different if the contour of one part differs greatly from the contour of the other, because the soil factors associated with differences in depth and texture will be different. If the land is fairly level and the soil uniform, however, the relative percentage-difference in yield $\left(\frac{x-y}{x} \times 100\right)$ of two plots treated alike will generally not exceed 5 to 10 per cent., which limit for the purpose of the type of investigation we are considering in this paper is sufficiently close to discover and elucidate rules or principles.

Definition of foliar diagnosis

Foliar diagnosis is characterized (**18**) as (a) the chemical composition of a leaf with respect to the dominant nutritive mineral entities at the instant of sampling, taken from a predetermined and suitable position on the stalk. (b) The foliar diagnosis for any given season (year) then will consist of a sequence of chemical states (composition) as determined under (a) on different dates, *i.e.*, at different periods during the growth season. *The composition is based on the dry matter of the leaf without taking into consideration the weight of the dry material at each sampling or the number of leaves sampled from each plant.*

It is clear, therefore, that no physiological significance can be attributed to the foliar diagnosis of any one fertilizer treatment (plot) considered alone. The method is comparative just as the method of analysis of entire plants is comparative (**41**). *Foliar diagnosis considered independently of all other field data and of all other foliar diagnosis has no physiological significance.*

Proper form to express mineral element content.—The criticism by Bartholomew, Watts, and Janssen (**2**) of the method used by the

writer of expressing the mineral content of a leaf on the basis of percentage of the element in the dried material is the result of misunderstanding and a confusion of thought. As JAMES (**12**) has well emphasized, the different ways of expressing the mineral content of plants in studies of mineral nutrition must be considered in relation to suitability. Each case must be considered on its merits. In the present instance, there can be no ambiguity nor question that the dry weight unit is the correct one. Furthermore, it is incorporated in the definition of foliar diagnosis (p. 573).

NOMENCLATURE

In investigations concerned with the mechanism of penetration into and translocation within the plant the logical procedure is to refer to all results in terms of the ions K, Ca, Mg, NO_3, H_2PO_4, etc. In physiological investigations having a direct agronomical application, this method is not the most suitable one. The facts are that in practical agriculture and in the arts dependent upon it, a different mode and only one mode of expression is internationally used for the dominant fertilizer principles or entities. These are N for nitrogen, P_2O_5 for phosphoric acid, and K_2O for potash. The writer has adopted this international nomenclature in this paper. And, as a matter of convenience, reference will frequently be made to the three dominant principles as "elements" or "entities." This should cause no confusion if the reader keeps in mind that all results are expressed as N, P_2O_5, and K_2O.

TWO METHODS OF EXPRESSING FOLIAR DIAGNOSIS GRAPHICALLY

The graphic presentation of the results of the periodic analyses of the leaves is important in the method of interpretation and may be done in one of two ways: (1) In terms of the percentage of the respective elements in the dried foliage as the ordinate and with the dates of sampling as the abscissa. (2) If only nitrogen, phosphoric acid, and potash are to be considered, the results may also be expressed graphically in trilinear coordinates.

The advantage of the first-mentioned method is that it possesses the capacity of expressing any number of elements for which analysis has been made and of showing the course of each independently. The second method is limited to three entities, in this case to nitrogen, phosphoric acid, and potash—elements which may properly be correctly considered as a unit of NPK, inasmuch as the content of all three in the dry foliage decreases with the age of the leaf, differing in this respect from Ca and Mg which accumulate with age.

BASIC ASSUMPTION IN FERTILIZER PLOT EXPERIMENTS.—The development and yield of a plant is, as already emphasized (p. 571), not exclusively dependent on nitrogen, phosphorus, and potassium. In drouth years water

would be the principal factor influencing yields. This factor and others associated with differences in soil texture will also enter in if the soil of the plots is not uniform. Consequently, there can be no *a priori* right to relate the development and yield to the composition of the leaf in terms of nitrogen, phosphoric acid, and potash. Nevertheless, fertilizer field-plot experiments are based on the assumption that the other factors for the plots to be compared are nearly equal. When they are not, recourse is had to many replicates. The present trend is to plan field plots in such a way that each experiment gives a measure of its own error, although, of course, they can never be exact.

Quantitative index expressing effect of a fertilizer entity

It has been a tradition to define the response to different fertilizer treatments in terms of the fertilizer or salts used. This point of reference has been used not only by the practical agriculturists but also by mathematicians (**7**), physiologists (**8**), and by agronomists generally. But when the fertilizer (or nutrient salt) treatment is used as the point of reference it is difficult to ascertain whether the effect is due to the sum of the individual elements (ions) or to an effect proportional to their product or even to a more complicated function of their individual effects (**7, 8, 14**).

If, however, a more rational base of reference be taken, *viz.*, the composition of the plant or better its "reflect," *viz.*, a leaf suitably chosen, the problem is considerably simplified. On this basis we may define without ambiguity the quantitative index expressing the effect of any one fertilizer element at the moment of sampling the leaves from any one plot, as the ratio of the amount of that element contained in the leaves of the plant growing on the plot considered, to the amount of that element in the homologous leaves from a plant growing on a plot receiving no fertilizer, sampled at the same time. Likewise, the quantitative index, expressing the effect of any combination of the elements present in the fertilizer used in any plot at the moment of sampling the leaves, may be defined as the ratio of the sum of the elements considered, contained in the leaves of plants growing on this plot at the moment of sampling, to the sum of these elements in morphologically homologous leaves of plants growing on the unfertilized plot.

Concepts of quantity and quality of nutrition

Inherent in the expression of the analyses in terms of the dried foliage are two concepts, *viz.*, that of *quantity* and *quality* of the mineral constituents. The concept of *quantity* or intensity of nutrition of the selected leaves consists of the sum $(N + P_2O_5 + K_2O)$ of each element at the moment of sampling expressed as a percentage of the dried material. The *quality* of nutrition is the ratio of these entities to each other at the moment of sampling.

Composite NPK-unit

Expressing the three dominant fertilizer principles as a unit.—The quantities of nitrogen, phosphoric acid, and potash in the dried foliage are expressed as a unit (**23, 24**) as follows:

Let x be the percentage of N, y the percentage of P_2O_5, and z the percentage of K_2O in the dry material of the leaf at the moment of sampling.

Let

$$s = x + y + z. \tag{1}$$

Then s denotes a quantity of mineral nutrients supplied by N, P_2O_5, and K_2O in proportion to the amounts x, y, and z present in the leaf considered at the moment of sampling. Dividing by s we get

$$1 = \frac{x}{s} + \frac{y}{s} + \frac{z}{s}. \tag{2}$$

where $\frac{x}{s}$, $\frac{y}{s}$, and $\frac{z}{s}$ represent fractions which indicate the proportion contributed by N, P_2O_5, and K_2O, respectively, to a unit quantity of the three elements. Let $\frac{x}{s}$ be denoted by x_1, $\frac{y}{s}$ by y_1, and $\frac{z}{s}$ by z_1. Substituting in (2), we get

$$x_1 + y_1 + z_1 = 1. \tag{3}$$

The quantity $x_1 + y_1 + z_1$ represents the composition of the leaf in terms of the proportions in which nitrogen, phosphoric acid, and potash are present in unit quantity of the leaf considered at the moment of sampling and, consequently, it is an expression giving the physiological ratios between N, P_2O_5, and K_2O in the synthetic laboratory of the leaf. It should be emphasized that this unit is independent of the total quantity s of N, P_2O_5, and K_2O in the dry material of the leaf. *The quantity "s," therefore, is a magnitude expressing the quantity of nutrition at the moment of sampling the leaf.*

The magnitude denoted by s indicates the number of units of nitrogen, phosphoric acid, and potash contained in 100 parts of dry matter and, consequently, s expresses the *quantity* (intensity) of nutrition with respect to nitrogen, phosphorus, and potassium. At first blush it might not seem justified to combine into one unit quantities as heterogeneous as N, P_2O_5, and K_2O. However, since the chemical reactions involved are not considered in this expression, but only the amounts of these fertilizer principles in the leaf irrespective of their chemical rôle, and inasmuch as the entities N, P_2O_5, and K_2O are those used in practical agriculture, it would be incongruous to express the characteristic *intensity of nutrition* in milligram equivalents.

Although these two magnitudes representing the *quality* and *quantity*

of nutrition are abstractly distinct, they are inseparably connected in the physiological processes of the leaf. They must, therefore, be considered simultaneously when relating the nutrition with respect to NPK of a leaf and its physiological effect on the development of the plant.

DIAGRAMMATIC ILLUSTRATION OF NPK-UNIT CONCEPT.—These concepts may be illustrated in the following manner (**25**).

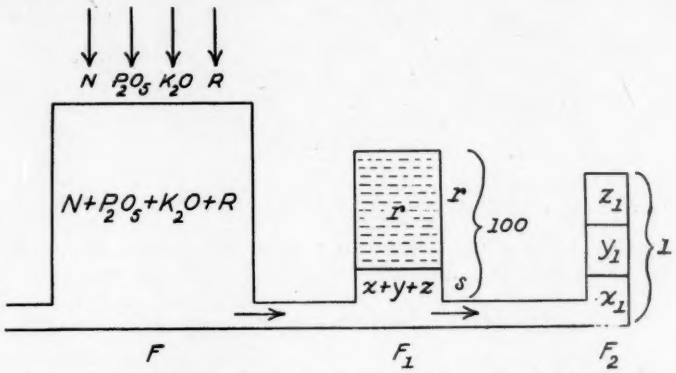

FIG. 1. Diagrammatic representation of the concept of the method of foliar diagnosis.

In figure 1 let F represent a leaf into which are migrating substances containing N, P, K plus other materials (R). Of the substances entering the leaf a portion will be elaborated, and a portion will remain in an unelaborated form. Let the quantities of all nutrients present at the particular instant of sampling a leaf be represented by a communicating vessel F_1 of capacity 100, of which $x+y+z=s$ represents the quantities of $N+P_2O_5+K_2O$ in 100 parts of the dried foliage and r the other substances present also in percentage of the dried foliage. F_1 represents the type of investigation with which foliar diagnosis is concerned. F_1 may then be regarded as an indicator of s.

Let us now imagine another vessel (F_2) *of unit capacity* to be connected with F_1, completely filled at the instant considered, and into which only N, P_2O_5, and K_2O are entering and in the same proportion as they are present in F and, consequently, also in F_1. Let us also suppose that N, P_2O_5, and K_2O are separated in F_2 into three layers as shown in the diagram. Then the sum of the quantities x_1, y_1, z_1 of nitrogen, phosphoric acid, and potash, respectively, of F_2 is always equal to 1. The vessel F_2 may then be regarded as the indicator of x_1, y_1, z_1, where $x_1=\frac{x}{s}$, $y_1=\frac{y}{s}$, and $z_1=\frac{z}{s}$.

CALCULATION OF NPK-UNIT IN TERMS OF MILLIGRAM EQUIVALENTS.—The *quality* factor of nutrition, *i.e.*, the proportion (ratio) of elements

found in the leaf at the respective sampling dates, is most suitably expressed as milligram equivalents (M.E.), because this factor—quality—must be dependent upon and related to the chemical reactions involved. These are calculated as follows:

Let M_x be the percentage of N, M_y the percentage of P_2O_5, and M_z the percentage of K_2O in the leaf at the moment of sampling. Then, reducing to milligram equivalents:

$$E_x = 1000\frac{M_x}{N};\quad E_y = 1000\frac{M_y}{\frac{1}{6}(P_2O_5)};\quad E_z = 1000\frac{M_z}{\frac{1}{2}(K_2O)}.$$

Let
$$S = E_x + E_y + E_z. \tag{4}$$

Dividing by S, we get

$$1 = \frac{E_x}{S} + \frac{E_y}{S} + \frac{E_z}{S}. \tag{5}$$

Then $\frac{E_x}{S}, \frac{E_y}{S}, \frac{E_z}{S}$, respectively, represents a fraction which indicates in milligram equivalents the proportional parts of N, P_2O_5, K_2O in a unit quantity of the three elements.

Let $\frac{E_x}{S}$ be denoted by x^1, then $E_x = x^1S$

" $\frac{E_y}{S}$ " " " " y^1, " $E_y = y^1S$

" $\frac{E_z}{S}$ " " " " z^1, " $E_z = z^1S$

$$x^1 + y^1 + z^1 = 1. \tag{6}$$

The quantity $x^1 + y^1 + z^1$ represents the composition of the NPK-unit of the leaf at the moment of sampling in terms of milligram equivalents.

To avoid fractional quantities it is convenient to multiply the terms of equation (6) by 100. Then

$$100\,x^1 + 100\,y^1 + 100\,z^1 = 100.$$

Let
$$X = 100\,x^1,\quad Y = 100\,y^1,\quad Z = 100\,z^1.$$

Then
$$X + Y + Z = 100. \tag{7}$$

$X + Y + Z$ is denoted the composite NPK-unit or, for simplicity, the NPK-unit. For each simultaneous value of X, Y, Z there is one point and only one on an equilateral triangle each of whose sides = 100.

READING THE TRILINEAR COORDINATE GRAPHS.—The higher the locus of a coordinate point moves in the direction of the summit of the triangle representing N = 100 per cent. in the *NPK-unit*, the greater is the proportion of nitrogen in this unit. Likewise, the movement of the locus of a coordinate point toward the left base-apex of the triangle representing $K_2O = 100$ per

cent. in the *NPK-unit*, the greater the proportion of potash in this unit. Similarly, changes in the direction of the locus of a point towards the right base-apex of the triangle shows that P_2O_5 in the *NPK-unit* has increased.

Application of method

Plots with treatments used

The potato plants used in this investigation were grown on tier 1, section B, of the Vegetable Fertility Plots of the Department of Horticulture. These plots were laid out in 1916. The description of the experimental lay-out given by Mack (**29**) is in part as follows:

"The area is laid out in four sections, 245.8 feet wide and 308.4 feet long. . . . Each of the sections is divided into six tiers. . . . The tiers are further divided into seventeen plots, the dimensions of which are 12 by 36.3 feet, and which are separated from each other by guard strips 6 feet wide. The area of the plots, exclusive of guard strips, is exactly 1/100 of an acre."

The four-year rotation consists of cabbage, potatoes, tomatoes, and sweet corn with timothy and rye as a green-manure crop.

The plots examined in this paper together with their treatments and yields (in 1935) are given in table I.

TABLE I

Plots with numbers and treatments

Tier	Plot no.	Commercial fertilizer applied	Commercial fertilizer applied to plot	N, P_2O_5, and K_2O equivalent	Symbol used	Yields per plot
			lb.	*lb.*		*lb.*
1	2	Sodium nitrate	4.0	0.6	N	109
1	3	Superphosphate	6.25	1.0	P	114
1	4	Potassium chloride	1.666	0.8	K	155
1	6	Sodium nitrate Superphosphate	4.0 6.25	0.6 1.0	NP	124
1	7	Sodium nitrate Potassium chloride	4.0 1.666	0.6 0.8	NK	163
1	8	Superphosphate Potassium chloride	6.25 1.666	1.0 0.8	PK	148
1	10	Sodium nitrate Superphosphate Potassium chloride	4.0 6.25 1.666	0.6 1.0 0.8	NPK	162

The complete fertilizer then has a ratio of $N:P_2O_5:K_2O$ of 0.6 : 1.0 : 0.8 or 6 : 10 : 8.

The plots of tier 1, section B, examined in this experiment were relatively uniform within the limitations defined on page 573. These plots represent only a few of the fertilizer combinations used in these crop fertility experiments. Other amounts with the same and also with different ratios are applied to the remaining plots. The best treatments reported in this paper, *viz.*, NK and NPK, are not those which have given the highest yields in this long continued experiment. The highest yield obtained with commercial fertilizers has been secured by a complete fertilizer containing the same quantity of nitrogen and potash, but containing 50 per cent. more phosphoric acid.

Selection and sampling of leaves

Ideal sample.—The ideal sample at any selected sampling date would consist of leaves of the same rank—(physiological or metabolic age)—on the stalks taken at the same time from a sufficient number of plants in a row along the plot to give a sample representative of the plot. Each successive row would then be sampled in like manner on each of the successive sampling dates. The leaf chosen from a plant would conveniently be the one immediately above the caducous leaves at the base of a stalk.

Sampling in relation to senescence.—Under practical field conditions one must determine beforehand the rank of the leaf or leaves to be selected, for, as explained earlier in this paper (pp. 573, 574), the method consists essentially of a comparison of the mode of nutrition of leaves of the same metabolic or physiological age from plants growing on plots subjected to different factors. To this end the longer the period of observation the greater will be the information obtained with respect to the differences in the course (nature) of the nutrition in response to the different treatments. If two plots receiving different treatments are planted the same day, the plants growing thereon will not reach maturity at the same time. At any intermediate date the plants considered will be at different stages of their life cycle, which can be determined in a general way by observation. With the qualification to be made later, the shorter-lived plants will show the greater amount of color change—from green to yellow and then brown. In the case of the potato leaf a stage is reached at which a golden yellow appears, followed by a further and final color change when it shrivels and becomes a dark brown and shortly after which it drops from the stem. These color changes are in general an indication of their metabolic age and the proportion of yellow and withered leaves on the plant is an index of condition.

Causes of senescence.—The aging of leaves has been associated with the disappearance of potassium (**3, 5**), of nitrogen (**4**), of water (**6**), and of all three elements, N. P, and K, simultaneously (**36**). The fact that an in-

creased supply of K has been found to delay breakdown of the mechanism causing aging (**11**) and that at higher concentrations this balance is under certain conditions upset, when yellowing becomes as fast or faster than with a deficiency (**11**), indicates that the mechanism is conditioned by physiological balance. In many instances, cause cannot be distinguished from effect (**3**).

METHOD OF SAMPLING.—Only under the practical field conditions of potato growing, where plants are planted in rows relatively close together and are well nourished, is the problem of sampling in relation to senescence of much moment. In such plants leaves higher up than the second and third may commence to wither before the end of the alloted sampling dates. This difficulty may be overcome in one of two ways. The one would be to take samples of the second or (and) third leaf from a stalk at short intervals of time. Then when the plants are sufficiently developed to take *simultaneously* samples of higher rank on the stalk, *e.g.*, the seventh and eighth leaf, and finally, as the plant matures, leaves still higher, *e.g.*, the twelfth or thirteenth leaf. This method of sampling has the advantage of giving three sets of overlapping samples which, if the plants are not subjected to too violent external disturbances, may be regarded as of corresponding successive physiological ages. The three sets of samples must then be treated separately in reporting results. This method will be illustrated in a later paper.

The second method of overcoming the difficulty is to sample a leaf from a particular position higher on the stalk, *e.g.*, the fourth or fifth leaf, which would give reasonable assurance of enabling observations to be made, say at three or four dates at intervals of about 12 to 20 days. The exact interval between samplings is not important. In the case of the potato plant the writer has found that there is at any given intermediate stage of maturity a progressive increase in nitrogen and phosphoric acid and a progressive decrease in potash of the dry matter of the leaf as one proceeds from the base upward, *i.e.*, the younger the leaf is. (*Cf.* MACGILLIVRAY, **28**, JAMES and PENSTON, **13**, and PENSTON, **32**.)

Accordingly, in the latter method of sampling there will be a slight progressive shift in rank on the stalk of the leaf of that rank with time. However, the differences expressed as percentages of the dried foliage with respect to N, P_2O_5, and K_2O of two consecutive leaves on the stalk of all cultivated plants thus far investigated have been found to be relatively insignificant compared with the differences between the composition of leaves of the same or approximately the same physiological age from plots receiving different treatments. This has been found to be true for any two successive leaves of the potato plants in these experiments. Such differences, therefore, for the purpose of this type of inquiry may be neglected.

Diurnal variations exist in the content of nitrogen, phosphoric acid, and

potash in the leaves of the potato (**33**). The magnitude of these diurnal variations is also insignificant compared with the magnitude of the changes considered in the method of foliar diagnosis. Samples, however, should be taken during the same day.

In the present experiments leaves were sampled on four different dates during July and August, and from consecutive rows on each successive date from two stems issuing from the mother tuber from each of the fourteen plants in a row. The number of samplings is arbitrary but at least three samplings should be made. The number of leaves sampled is also immaterial but, obviously, should not vary too greatly from one plot to another. The development of the plants sampled from each plot should be regular and homogeneous, for unless this is the case there would be no justification for considering the samples as representing an average sample.

It should now be clear that foliar diagnosis must be expressed in terms of the rank of the leaf on the stem, the variety of plant, and the year in which samples were taken.

Removal of spray residues, soil, and dust from leaves

The spray materials used contained calcium (and magnesium). It is desirable to remove as much spray material from the leaves as possible, including any soil and dust adhering thereto, so as not to affect the weight of the dry material taken for analysis. The soil and dust are easily removed and most of the spray materials also by brushing with a stiff brush in the field immediately after the samples are taken.

Method of drying leaves

The leaves were dried in a drying oven at 100° C. after being brought in from the field and then finely ground in a Wiley mill.

Determination of nitrogen, phosphoric acid, and potash

Nitrogen was determined by the Kjeldahl-Gunning method to include the nitrogen of nitrates (**1**), phosphoric acid by the Richards and Godden's (**35**) modification of the Pemberton-Neumann (**30, 31**) method, and potash by the Lindo-Gladden method (**1**).

Presentation of results

Table II gives the results of the analysis of the fourth and fifth leaves expressed in (a) percentage of N, P_2O_5, and K_2O in the dried foliage, (b) milligram equivalents, and (c) the NPK-unit.

Calculation of loci of NPK-unit

The following illustrates the method of calculating a coordinate point:

TABLE II

COMPOSITION OF LEAVES OF RANK 4 AND 5 AT PERIODIC INTERVALS IN TERMS OF PERCENTAGE OF N, P_2O_5, AND K_2O IN DRIED FOLIAGE, MILLIGRAM EQUIVALENTS, AND THE NPK-UNIT

DATE OF SAMPLING	DRIED FOLIAGE			MILLIGRAM EQUIVALENTS				COMPOSITION OF THE NPK-UNIT		
	N	P_2O_5	K_2O	N	P_2O_5	K_2O	$E_x + E_y + E_z$	X	Y	Z
	(M_x)	(M_y)	(M_z)	(E_x)	(E_y)	(E_z)	(S)	$\left(100\frac{E_x}{S}\right)$	$\left(100\frac{E_y}{S}\right)$	$\left(100\frac{E_z}{S}\right)$
	%	%	%	*mg. eq.*	*mg. eq.*	*mg. eq.*	*mg. eq.*	%	%	%
					N plot (tier 1, no. 2)					
July 7	5.12	.404	3.97	365.568	17.089	84.561	467.218	78.244	3.658	18.098
July 29	4.42	.534	2.54	315.388	22.588	54.102	392.278	80.450	5.759	13.791
Aug. 9	4.15	.472	2.01	296.310	19.966	42.813	359.089	82.517	5.561	11.922
Aug. 24	3.64	.344	1.77	259.896	14.551	37.701	312.148	83.261	4.661	12.078
					P plot (tier 1, no. 3)					
July 7	4.57	.550	2.41	326.298	25.042	51.333	402.673	81.034	6.218	12.747
July 29	3.46	.521	1.64	247.044	22.038	34.932	304.014	81.260	7.250	11.490
Aug. 9	3.24	.520	1.29	231.336	21.996	27.477	280.809	82.383	7.831	9.786
Aug. 24	2.79	.450	1.11	199.206	19.035	26.643	241.884	82.359	7.868	9.773
					K plot (tier 1, no. 4)					
July 7	4.61	.424	6.32	329.154	17.935	134.573	481.662	68.336	3.725	27.939
July 29	3.87	.540	5.22	276.318	22.842	111.122	410.282	67.349	5.567	27.084
Aug. 9	3.39	.464	4.91	242.046	19.627	104.689	366.362	66.068	5.358	28.575
Aug. 24	2.78	.370	5.00	198.492	15.651	106.500	320.643	61.904	4.881	33.215

TABLE II—(*Continued*)

Date of sampling	Dried foliage			Milligram equivalents				Composition of the NPK-unit		
	N	P_2O_5	K_2O	N	P_2O_5	K_2O	$E_x+E_y+E_z$	X	Y	Z
	(M_x)	(M_y)	(M_z)	(E_x)	(E_y)	(E_z)	(S)	$\left(100\frac{E_x}{S}\right)$	$\left(100\frac{E_y}{S}\right)$	$\left(100\frac{E_z}{S}\right)$
	%	%	%	*mg. eq.*	*mg. eq.*	*mg. eq.*	*mg. eq.*	%	%	%
					NP plot (tier 1, no. 6)					
July 7	5.14	.590	2.53	366.996	24.957	53.910	445.863	82.311	5.598	12.091
July 29	3.87	.532	1.52	276.318	22.504	32.355	331.177	83.435	6.794	9.771
Aug. 9	3.71	.486	1.19	264.894	20.558	25.262	310.714	85.253	6.617	8.130
Aug. 24	3.36	.390	1.09	239.904	16.751	23.110	279.765	85.752	5.987	8.261
					NK plot (tier 1, no. 7)					
July 7	5.10	.464	6.63	364.140	19.627	141.176	524.943	69.367	3.739	26.894
July 29	4.03	.510	5.55	287.742	21.573	118.300	427.615	67.290	5.045	27.665
Aug. 9	3.78	.452	5.28	269.892	19.119	112.443	401.454	67.229	4.763	28.008
Aug. 24	3.26	.352	5.46	232.764	14.889	116.234	363.887	63.964	4.092	31.941
					PK plot (tier 1, no. 8)					
July 7	4.55	.620	6.76	324.870	26.395	143.967	495.232	65.599	5.330	29.071
July 29	3.36	.510	5.32	239.904	21.573	113.337	374.814	64.006	5.755	30.239
Aug. 9	3.08	.490	5.35	219.912	20.980	113.912	354.804	61.981	5.913	32.105
Aug. 24	2.82	.430	5.04	201.348	18.189	107.309	326.846	61.603	5.565	32.832
					NPK plot (tier 1, no. 10)					
July 7	4.98	.584	6.59	355.572	24.703	140.346	520.621	68.297	4.744	26.958
July 29	3.88	.509	4.34	277.032	21.531	92.378	390.941	70.863	5.507	23.630
Aug. 9	3.62	.482	4.70	258.468	20.389	100.046	376.903	68.215	5.381	26.405
Aug. 24	3.14	.392	4.35	224.196	16.582	92.612	333.390	67.249	4.973	27.778

The analytical results (table II) for the N plot (no. 2) sampled July 7 are N = 5.12 per cent., P_2O_5 = 0.404 per cent., and K_2O = 3.97 per cent.

The *intensity of nutrition* (s) of the selected leaves for the N plot on July 7 is, therefore,

$$N + P_2O_5 + K_2O = 5.12 + 0.404 + 3.97 = 9.494$$

To evaluate the ***NPK-unit*** the percentage values are converted (pp. 577, 578) into milligram equivalents (M.E.) of N, P_2O_5, and K_2O as follows:

$$E_x = 1000 \times 0.0714 \times 5.12 = 365.568 \text{ M.E. of N}$$
$$E_y = 1000 \times 0.0423 \times 0.404 = 17.089 \text{ M.E. of } P_2O_5$$
$$E_z = 1000 \times 0.213 \times 3.97 = 84.561 \text{ M.E. of } K_2O$$

Hence

$$S = E_x + E_y + E_z = 365.568 + 17.089 + 84.561 = 467.218$$

and

$$X = 100\,\frac{E_x}{S} = \frac{100 \times 365.568}{467.218} = 78.244$$

$$Y = 100\,\frac{E_y}{S} = \frac{100 \times 17.089}{467.218} = 3.658$$

$$Z = 100\,\frac{E_z}{S} = \frac{100 \times 84.561}{467.218} = 18.098.$$

These values give the three coordinate points required. It should be noted that 78.244 + 3.658 + 18.098 = 100. *We then have for each simultaneous value of X, Y, Z a representative point and only one upon the surface of an equilateral triangle the sides of which are expressed in 100 units.*

Meteorological conditions during 1935 growing season

The foliar diagnosis specified in this paper is that for the fourth and fifth leaves for the potato variety Rural Russet for the year 1935. As a matter of record the meteorological conditions (rainfall and mean daily temperature) for the month preceding the sampling and for the intervals between samplings are given in table III.

TABLE III

Record of meteorological conditions

Date	Rainfall	Mean daily temperature
	in.	°*F.*
June 1–30	3.88	66.5
July 6–29	5.67	73.5
July 30–Aug. 9	1.19	70.8
Aug. 10–24	0.35	71.4

Interpretation and discussion of results

Indications given by first method of graphic presentation based on percentages of elements in dried foliage as ordinate and dates of sampling as abscissa

When the percentage of an element in the dried foliage at a particular sampling date is plotted as the ordinate and the dates of sampling as the abscissa, as described in the first method (p. 574), the relationships between the composition of the leaves, with increasing age, from plants growing on the respective plots are clearly brought out and are shown in figure 2.

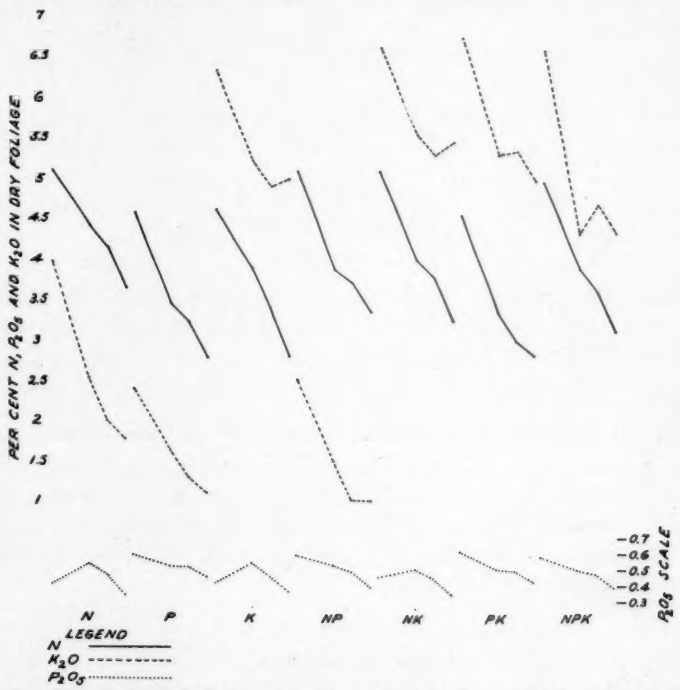

FIG. 2. Periodic analysis of the fourth and fifth leaves of potato plants growing on the differently treated plots showing percentage of elements in the dried foliage as ordinates and the dates of sampling as abscissa.

The indications given by the graphs are summarized below:

NITROGEN GRAPHS.—The content of nitrogen expressed as percentage in the dried foliage of plants which received mineral nitrogen applications is higher throughout the whole period than in those which received none.

Furthermore, the nitrogen content decreases with the increasing age of the leaf throughout the whole period in all plots.

PHOSPHORIC ACID GRAPHS.—The content of phosphoric acid expressed as a percentage in the dried foliage from plants which received phosphate applications is higher throughout the whole period than in those which received none. The phosphoric acid content decreases with the increasing age of the leaves of plants growing on plots to which mineral phosphate was added; but in the leaves of all plants which did not receive mineral phosphate additions a maximum occurs at the second sampling (July 29), after which the content of P_2O_5 again decreases regularly with the increasing age of the leaf. This accumulation is undoubtedly a temperature effect. The curve of mean daily temperature is an ascending one up to July 24, after which it begins to descend. This temperature effect would, however, be masked in the plots where phosphorus is rapidly utilized.

POTASH GRAPHS.—The content of potash expressed as a percentage in the dried foliage of all plants which received mineral potash applications is very much higher than in those which did not. The potash content decreases regularly throughout the whole period with increasing age of the leaves of plants growing on plots which did not receive mineral potash. But accumulation has occurred in the leaves from all plants growing on plots which received mineral potash applications. This accumulation commenced in some cases (NPK, no. 10; PK, no. 8) between July 29 and August 9 and in others (NK, no. 7; K, no. 4) between August 9 and August 23.

JAMES (**12**) concludes that in the potato plant potassium is eventually heaped up considerably in excess of the amounts which are necessary to maintain the declining rate of growth. The data presented above show that generalizations with respect to accumulation of potassium are not possible. This accumulation is the result of a lack of physiological balance. It will be shown in a later paper that this accumulation of potassium has not occurred in the leaves of plants growing on plots which received sufficient nitrogen and phosphoric acid for the utilization of the potash.

It is thus seen that periodic analysis of leaves of the same physiological age from plants growing on plots treated with different fertilizers reflects these additions. This also holds true for plots on tier 2 and their duplicates on tiers 4 and 5. The graphs show by their position and their form the nature or course of the nutrition with respect to nitrogen, phosphorus, and potassium in the plants growing on the different plots. The steepness (slope) of a graph of a particular element indicates the relative demand in relation to the supply of that element.

Certain other indications furnished by the data given in table II are best brought out by the second method described on pages 576 and 577. In this method nitrogen, phosphoric acid, and potash are evaluated as a unit-NPK

and the results are plotted in trilinear coordinates. This method is illustrated in figures 4, 5, 6, and 7.

Indications given by second method of graphic presentation in which results are expressed as NPK-units and plotted in trilinear coordinates

In interpreting these graphs (figs. 4, 5, 6, 7) it should be kept in mind that a fertilizer may intervene to produce either (1) an increase in the sum ($N + P_2O_5 + K_2O$), *i.e.*, in the *intensity of nutrition*, or (2) a change in the composition of the *NPK-unit*, or (3) a change in both (1) and (2) simultaneously.

The *intensities of nutrition* are shown in figure 3 with the percentage of

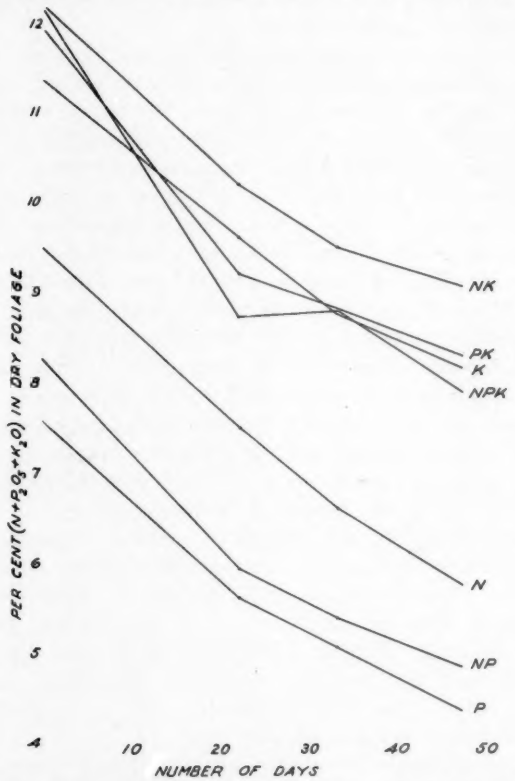

Fig. 3. Relationships of the intensities of nutrition of plants growing on differently treated plots.

TABLE IV

RELATION OF MEAN NPK-UNIT AND MEAN INTENSITY OF NUTRITION AT FOUR SAMPLING DATES TO YIELDS OF POTATOES ON VARIOUS PLOTS

TREATMENT	TIER	PLOT	MEAN NPK-UNITS (MEAN OF 4 SAMPLINGS)			INTENSITY OF NUTRITION (MEAN OF 4 SAMPLINGS)	YIELDS		
			N :	P_2O_5 :	K_2O		POUNDS PER PLOT	RELATIVE YIELDS NK = 100	CLASS ORDER
			%	%	%		*lb.*	%	
NK	1	7	66.92 :	4.28 :	28.61	10.24	163	100.00	1
NPK	1	10	68.65 :	5.15 :	26.19	9.39	162	99.38	
K	1	4	65.91 :	4.88 :	29.20	9.48	155	95.09	2
PK	1	8	63.29 :	5.64 :	31.06	9.56	148	90.80	
NP	1	6	84.18 :	6.25 :	9.56	6.10	124	76.07	3
P	1	3	81.76 :	7.29 :	10.95	5.65	114	69.99	
N	1	2	81.12 :	4.91 :	13.97	7.34	109	66.93	

$N + P_2O_5 + K_2O$ in the dried foliage from plants growing on the respective plots as the ordinate and as abscissa the dates of sampling. The coordinate points for the respective sampling dates, July 7 and 29, and August 9 and 24, are shown by dots. The displacement in a particular graph from one sampling date to another indicates the changes in the *intensity of nutrition* with the age of the leaves of the plants growing on the plot considered; and the relationship in form and position of the graphs to one another indicates the relative intensities of nutrition (s) resulting from the different treatments.

In figures 4, 5, 6, and 7 are shown in trilinear coordinates the changes in the composition of the *NPK-unit* (*i.e.*, in the *quality of nutrition*) of the selected leaves from plants growing on the differently treated plots. The numbers 1, 2, 3, 4 at the respective coordinate points indicate the successive sampling dates, July 7 and 29, and August 9 and 24, respectively. The displacement of a point from one sampling date to another, therefore, indicates the course of the nutrition with respect to the NPK-unit, as previously defined (p. 578), and which represents the equilibrium between N, P_2O_5, and K_2O at the moment of sampling.

RELATIONSHIP BETWEEN FOLIAR DIAGNOSIS AND YIELDS OF TUBERS.—*The mean NPK-unit.*—Table IV gives (1) the mean NPK-units for each plot, *i.e.*, the mean NPK-unit for the four sampling dates of the selected

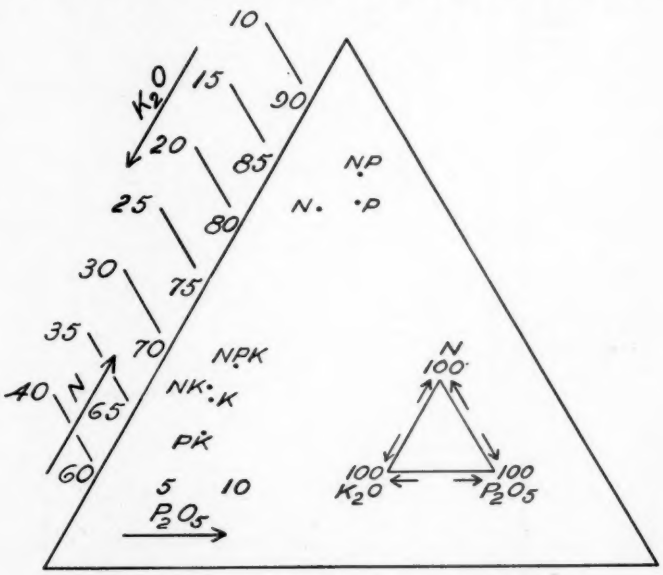

FIG. 4. Relative positions of the mean NPK-units.

leaves from plants growing on each plot; (2) the mean intensities of nutrition, and (3) the actual and relative yields of tubers on the basis of the highest yielding of the plots examined being placed at 100.

The mean NPK-*unit* for each plot is shown graphically in figure 4 by means of a single point for each plot. Each point, therefore, corresponds to the center of gravity (C.G.) of the detailed diagram shown in figures 5, 6, and 7 for the corresponding plot.

The plots are seen to fall into two groups according to their foliar diagnosis, *i.e.*, to their position on the triangle. The one group consisting of the N, P, and NP plots which are found high up toward the N = 100 per cent. apex of the triangle (*i.e.*, the corner representing N = 100 per cent.), and the other group the plots NK, NPK, K, and PK which are found much lower down in the triangle toward the left corner, *i.e.*, the corner representing K_2O = 100 per cent.

The latter group can be further subdivided into two groups according to the extent of their displacement toward the base of the triangle. The points representing, respectively, the mean NPK-unit of the plots K (no. 4) and PK (no. 8) are lower than the points representing the plots NK (no. 7) and NPK (no. 10). The groups are designated as follows:

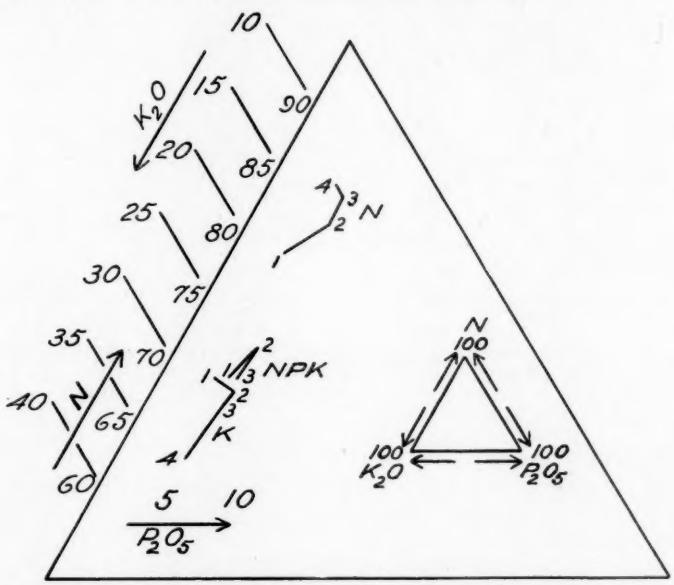

FIG. 5. Course of nutrition of plants growing on the N (no. 2), K (no. 4), and NPK (no. 10) plots as indicated by the changes in their NPK-units from one sampling date to another.

Group 3, NP (no. 6), P (no. 3), N (no. 2), having low yields.
Group 2, K (no. 4), PK (no. 8), having intermediate yields.
Group 1, NK (no. 7), NPK (no. 10), having high yields (the highest reported in this paper).

GRAPHS IN DETAIL.—The displacement of the *NPK-unit* from one sampling date to another for all treatments cannot be shown in one diagram, because of the confusion resulting from the intersection of the graphs from certain of the plots. The detailed graphs showing the course of nutrition are best presented by making use of three diagrams (figs. 5, 6, 7) so arranged that the graphs for the respective treatments are compared with the complete fertilizer plot.

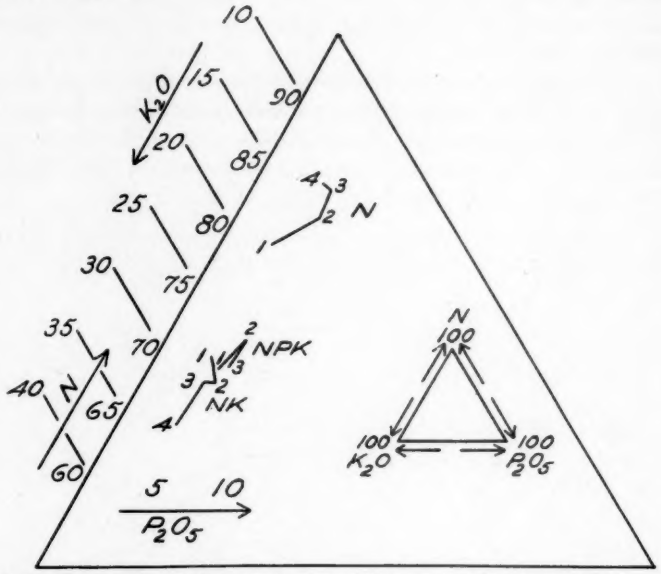

FIG. 6. Course of nutrition of plants growing on the N (no. 2), NK (no. 7), and NPK (no. 10) plots as indicated by the changes in their NPK-units from one sampling date to another.

Foliar diagnosis of low-yielding group 3—N (no. 2), P (no. 3), and NP (no. 6).—The *intensity of nutrition* is very low (range of the mean is 7.34 to 5.65). This low intensity is the result of the very low content of K_2O (see table II, column 3, for plots 2, 3, 6).

Nitrogen in the *NPK-unit* is very high (the range of the mean NPK-unit is 81.1 to 81.7). Potash is very low (the range of the mean NPK-unit is 13.9 to 9.56). Phosphoric acid is high in those which received phosphate additions, *viz.*, NP (no. 6) and P (no. 3).

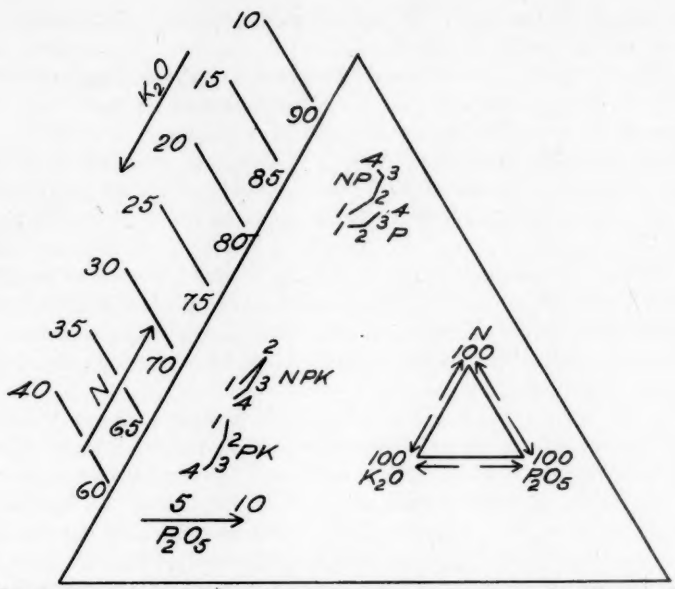

FIG. 7. Course of nutrition of plants growing on the P (no. 3), NP (no. 6), PK (no. 8), and NPK (no. 10) plots as indicated by the changes in their NPK-units from one sampling date to another.

Foliar diagnosis of intermediate-yielding group 2—K (no. 4) and PK (no. 8).—The mean intensity is 9.48 and 9.56, respectively, for the plants growing in the K plot (no. 4) and the PK plot (no. 8). In this group the intensity is slightly higher than that of the complete fertilizer plot (no. 10).

Nitrogen in the *NPK-unit* is much lower than in the low-yielding group 3 and also somewhat lower than in the higher-yielding group 1. The mean NPK-unit is 63.3 and 65.9 in PK (no. 8) and K (no. 4), respectively. Potash in the NPK-unit is higher than in both groups 1 and 3.

Foliar diagnosis of high-yielding group 1—NK (no. 7) and NPK (no. 10).—The mean intensity is 10.24 in NK (no. 7) and 9.39 in NPK (no. 10). The plants growing on plot NK (no. 7) have the highest intensity reported in this paper, but plants growing on three other plots in this tier (not reported in this paper) have still higher intensities. This high intensity of nutrition in NK (no. 7) relative to plants growing on the other plots is due to the phenomenon of the reciprocal effects of the elements (**40**). In this particular case the omission of phosphorus from the complete fertilizer has resulted in a much greater uptake of the elements remaining (compare columns 1, 2, 3 of table II for NK (no. 7) and NPK (no. 10).

Nitrogen in the *NPK-unit* is much lower and potash much higher than

in the low-yielding group 3. Phosphoric acid in the NPK-unit has the lowest value in NK (no. 7) of any plot.

Course (nature) of nutrition with respect to NPK-unit in group 3—N (no. 2), P (no. 3), and NP (no. 6).—The course of the nutrition (*mode d'alimentation*) in this low-yielding group is strongly contrasted. This group, however, has one characteristic in common, *viz.*, nitrogen in the *NPK-unit* continuously increases at the expense of the potash with the increasing age of the leaves, as shown by the upward displacement of the graphs at each successive sampling period. In N (no. 2) and NP (no. 6) this tendency is quite marked. Consequently, the application of nitrogen without potash has had a strong tendency to reverse the direction of the nitrogen nutrition which the medium (soil) has tended to impose. This tendency is also observed but to a smaller extent with phosphate applied alone (P plot, no. 3), *i.e.*, without mineral nitrogen applications.

It is also to be noted that in the leaves of plants growing on the N plot (no. 2) and which, therefore, has received no phosphate additions for nearly 20 years other than that added in the system of green manuring, the phosphorus in the *NPK-unit* increases with the age of the leaf up to the third sampling, after which a decrease occurs. The proportion of phosphorus in the *NPK-unit* of the N (no. 2) plot is not, however, as high as that in the leaves from plants growing on the NP (no. 6) or P (no. 3) plots which received mineral phosphates. The continual accumulation of phosphorus throughout the whole period in the NPK-*unit* of plants growing on Plot P (no. 3) is very marked, signifying poor assimilation of this entity (element) by the plants growing on this plot.

Course of nutrition with respect to NPK-unit in group 2—K (no. 4) and PK (no. 8).—The graphs indicate that the course of the nutrition with respect to the *NPK-unit* of the plants growing on these plots is very different throughout the whole period. The relative yields of tubers from plot K (no. 4) to that of plot PK (no. 8) on this tier 1 is 95.1 : 90.8, associated with which is the fact that nitrogen in the *NPK-unit* of plants growing on plot K (no. 4) is higher than are those of plot PK (no. 8) throughout the whole period. The omission of phosphorus, therefore, has on this particular tier resulted in increasing the nitrogen in the *NPK-unit* of plants growing on plot K (no. 4) from one (PK, no. 8) classed as too low in nitrogen. This is another example of the phenomenon of reciprocal effects (**40**).

Course of nutrition with respect to NPK-unit in group 1—NK (no. 7) and NPK (no. 10).—The omission of mineral phosphates has in this case also changed the character of the nutrition with respect to the *NPK-unit*. On July 7 the proportion of nitrogen in the *NPK-unit* in the leaves of plants growing on plot NK (no. 7) is greater than in those from the complete fertilizer plot (no. 10). But, whereas in the former (plot no. 7) the nitrogen in

the *NPK-unit* has continuously decreased with the age of the leaf, in the latter (plot no. 10) the nitrogen in the *NPK-unit* has rapidly increased at the expense of the potash during the first period (July 7 to 29) and on July 29 is greater than that of the NK plot. Furthermore, during this first period (July 7 to 29) the phosphorus in the NPK-*unit* of leaves of plants growing on the NK (no. 7) plot has increased much more rapidly than in those from the complete fertilizer plot (no. 10), although phosphorus always remains at a higher level in the NPK-*unit* of plants growing on plot no. 10 throughout the whole period.

The slightly greater increased yields of tubers from NK (no. 7) over those of the complete fertilizer plot (no. 10) in this tier may not be fortuitous. The causative factor may be a higher proportion of potassium in the *NPK-unit* of plants growing on plot NK (no. 7) made at the expense of the nitrogen. In other words, the physiological balance with respect to the dominant nutritive elements in plants growing on plot NK (no. 7) appears to be better than that on plot NPK (no. 10) in this particular tier. Further corroborative evidence is the fact that the *NPK-unit* of NK (no. 7) is nearer the *NPK-unit* of plants growing on the plot giving the highest yield on this tier, *viz.*, manure (plot no. 15).

Effect of omission of an element from complete fertilizer on NPK-unit.— The effects are summarized as follows:

1. The omission of nitrogen from the complete fertilizer has increased the potash and the phosphoric acid in the *NPK-unit* of the leaf throughout the whole period, resulting in decreased yields.

2. The omission of potash from the complete fertilizer has increased the nitrogen and the phosphoric acid in the *NPK-unit* of the leaf throughout the whole period, resulting in decreased yields.

3. The omission of phosphorus from the complete fertilizer has increased the potash in the *NPK-unit* of the leaf at the expense of the nitrogen, resulting in a slight increase of yield.

Effect of omission of two elements from complete fertilizer on NPK-unit.— The effects are summarized as follows:

1. The omission of both phosphorus and potash has increased the nitrogen in the *NPK-unit,* resulting in considerable decrease in yield.

2. The omission of both nitrogen and potash has increased the nitrogen and the phosphorus in the *NPK-unit* with considerable reduction in yields.

3. The omission of both nitrogen and phosphorus has resulted in increasing the potash in the *NPK-unit,* resulting in decreased yields.

We are attempting by means of foliar diagnosis to discover the influence of the soil and the weather on the action of fertilizers. It should be clear, therefore, that no generalization is made from these results with respect to response of potatoes on a Hagerstown clay-loam soil resulting from the treatments used. The foliar diagnosis and the corresponding yields are for the

plots indicated on tier 1, section B, of the Vegetable Fertility Plots in the year 1935. In later papers will be reported the relation of foliar diagnosis to optimum nutrition of the potato in the year 1935 and also the relationship of foliar diagnosis to other similarly treated (duplicate) plots in tiers 4 and 5 of this same section B.

Summary

1. The foliar diagnosis of a particular plant species at any given instant of time consists of the mineral composition of a leaf from a definite rank (metabolic age) on the stem. The succession of chemical states with respect to a leaf of the same rank of a particular species as determined at different dates characterizes the foliar diagnosis of that particular plant species (and variety) for the particular growth season examined. The composition is based on the dry material of the leaf without taking into consideration the weight or number of leaves.

2. The physiological facts upon which the method is established are described.

3. Two methods of expressing the results graphically are given and the indications given by each of them presented.

4. It is shown that inherent in the concept of foliar diagnosis is the concept of (1) *quantity* or intensity of nutrition, (2) *quality* of nutrition or the ratio of the dominant elements to one another.

5. The dominant nutritive elements (entities) nitrogen, phosphoric acid, and potash are expressed as a *unit of NPK*. This is designated the *NPK-unit*. The method of deriving the unit is described.

6. Potato plants of the variety Rural Russet growing in 1935 on experimental plots treated with the different combinations of commercial fertilizers were examined by the method. The plots reported in this paper consist of seven on tier 1, section B, of the long-continued Vegetable Fertility plots of the Pennsylvania Agricultural Experiment Station. The treatment consists of the following combinations of fertilizers: N, P, K, NP, NK, PK, NPK.

7. The succession of coordinate points of the *NPK-unit* for the fourth and fifth leaves from the base taken on July 7 and 29, and August 9 and 24, 1935, from plants growing on the differently treated plots are shown in trilinear coordinates. These graphs indicate (1) the course (nature) of the nutrition with respect to the *NPK-unit*, and (2) the relationship between the foliar diagnosis and the yields of tubers.

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UREASE DISTRIBUTION IN *CANAVALIA ENSIFORMIS*[1]

SAM GRANICK[2]

(WITH TEN FIGURES)

Introduction

In this account the distribution of urease, as studied with the histological and quantitative methods which were described in the first paper of this series (**2**), will be discussed under the subheadings of the various plant structures. The definition of some of the terms used are as follows:

Urease activity is expressed in urease units. A urease unit (U.U.) is a quantity of urease which will produce 1 mg. of ammonia per minute when acting upon urea under the experimental conditions employed (**2**). The indicator method is not as sensitive as the quantitative method, and tissues containing less than 0.02 U.U. per gram of fresh weight are reported as negative with this method. Tissues containing less than 0.0001 U.U. are reported as negative with the quantitative method.

The *hypocotyl* is that part of the axis between the cotyledonary node and the external collet.

The *external collet* is the region below the hypocotyl, bearing a considerable tuft of lateral roots.

The *first internode* is the hypocotyl.[3]

The *first node* is the cotyledonary node.

The *second node* is that part of the stem axis from which the first foliage leaves arise.

Canavalia ensiformis or jack bean is an annual legume usually bushy and erect, 1 to 2 meters high; the tips of the branches inclined to twine; peduncles stout, 10 to 20 flowered; pods linear, 25 to 30 cm. long; seeds ellipsoid, shiny white, $22 \times 14 \times 8$ mm. The plants grown in the greenhouse at Ann Arbor were 3 to 4 meters high and spindling. The distinctive taxonomical description of *Canavalia ensiformis* is given by PIPER (**5**). The gross morphology of the plant closely resembles that of *Phaseolus vulgaris* whose anatomy has recently been studied by DOUTT (**1**).

[1] Second of a series of three papers on urease distribution in *Canavalia ensiformis* and *Soja max*. The reader is referred to the thesis available at the University of Michigan library for more extensive data on urease in the jack bean, for descriptions of the relevant anatomical and embryological features of the plant, and for the literature on the presence of urease in various species of the plant kingdom.

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[3] This convenient designation, used solely for the purpose of this paper, is not technically correct according to morphological terminology which would consider the first internode as the region between the cotyledonary node and the first foliage leaves.

The bulk of the seed of jack bean consists of cotyledons which are made up largely of parenchyma cells with thickened hemicellulose walls possessing numerous tiny pits. SUMNER (**6**) estimates that the bean contains 0.12 to 0.06 per cent. urease and considers the crystalline urease to be globulin, because it is insoluble in the absence of neutral salts in the vicinity of its isoelectric point. About 25 per cent. of the air-dried seed is protein (**4**).

Experimentation

COTYLEDONS

Employing the histological urease methods, examination has shown that the cotyledons contain more urease than any other tissues of the plant. Studies of the seeds after two days of germination in sphagnum revealed that the enzyme concentration was greatest in the subepidermal cells, and especially in the bundle sheaths, and phloem tissue, that is, in those cells of the cotyledons containing the densest cytoplasm (fig. 1). The bundles of the cotyledons immediately adjacent to and merging with the conducting bundles of the hypocotyl appeared to contain less urease than the other bundles of the cotyledons. This may be due to a more rapid elongation of the bundles adjacent to the cotyledons, the enzyme thus becoming more diffuse, or it may be due to decomposition of the enzyme in the bundles. As germination proceeds, urease decreases rapidly in the epidermis and in the two to three layers of subepidermal parenchyma cells. The enzyme disappears from some parenchyma cells more rapidy than from others. No relation could be noted between the enzyme content of the cells close to the bundles, and those more or less isolated from the bundles. This suggests that there is no transport of active urease to the bundles. The cotyledons which fell off after 20 days still contained relatively large quantities of urease. Sectioning at this stage revealed that the cells were thin-walled with large vacuoles, and contained numerous chloroplasts but little starch.

TABLE I

UREASE CONTENT OF COTYLEDONS OF JACK BEAN SEEDLINGS

DAYS AFTER PLANTING	FRESH WT. PER COTYLEDON	DRY WT. PER COTYLEDON	U.U. PER GM. FRESH WT.	U.U. PER COTYLEDON
days	*gm.*	*gm.*		
0	0.651	0.560	.....	.....
1	1.05	0.561	31.2	32.8
3	1.25	0.532	25.9	32.5
5	1.15	0.456	24.4	28.0
7	1.26	0.377	12.6	15.9
10	1.17	.....	11.5	13.5
20*	0.483	.....	1.08	0.523

* The cotyledons had just fallen off; they were quite thick, yet flaccid.

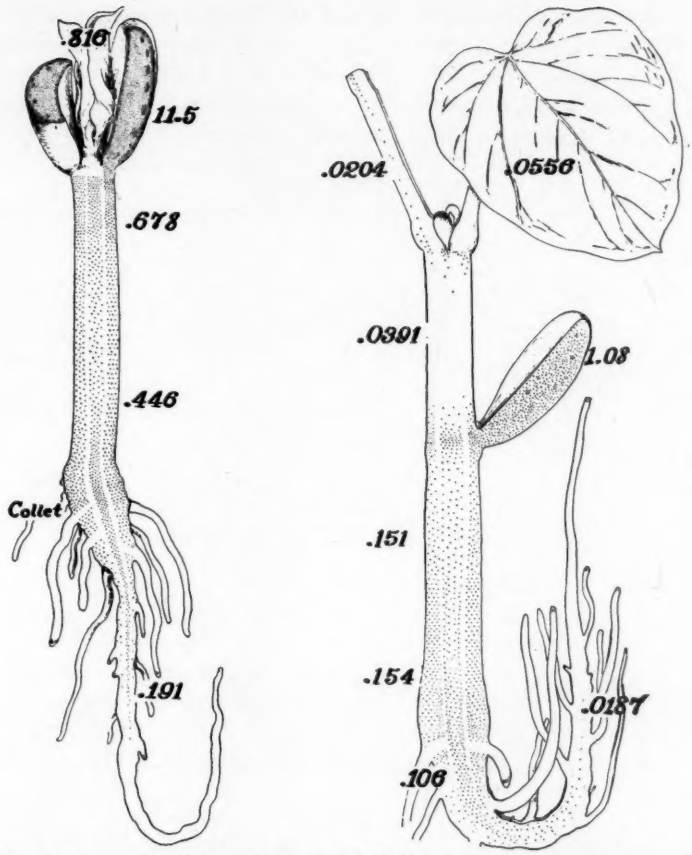

FIG. 1. Composite picture of urease distribution in the various structures of jack bean seedlings 10 and 20 days old. The figures opposite the various regions of the seedling represent the number of urease units per gram of fresh weight of the region, as determined by the quantitative method. Stippling indicates the relative urease concentration as revealed by the histological methods.

Quantitative studies were made of the urease content of cotyledons during the development of the seedlings. It will be noted from table I that the urease content of the cotyledon decreases rapidly as the seedling becomes older. The data are interpreted as indicating, not that the enzyme is gradually inactivated by poisons or other inactivators, nor that it is transported intact to other tissues, but that it is broken down just as are other storage proteins of these cells, into some soluble compounds which are then

transported to other plant parts. This viewpoint will be discussed later in more detail.

Radicle

In the radicle 1 cm. long (fig 2.) the histological reagent shows urease to be present in all of its parts except the primary xylem elements. The

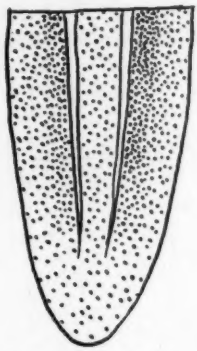

Fig. 2. Urease concentration in a radicle 1 cm. long.

inner cortical cells contain the most enzyme, the outer cortical cells and pith less enzyme, and the cells of the radicle tip still less. The dermatogen appears to contain little or no enzyme; it is, however, slightly more acid (pH 4.4) than the other tissues. The weak indicator-change in the dermatogen may possibly be due to the fact that more acid has to be neutralized before the indicator shows an alkaline reaction than in the other tissues. Therefore, a positive statement cannot be made concerning its relative urease content.

Cross-sections of a 3-cm. radicle reveal a decrease of urease in the region in which cell elongation has occurred, that is, at approximately 2 cm. distal to the tip. At this level there is less enzyme in the pith than in the cortex. After four days' germination the radicle has attained a length of 5 cm. and the collet region with its tuft of secondary roots has appeared.

Collet

The collet region was made the subject of particular study, since it is in this region that the various stages of lateral root differentiation can most readily be obtained. In 5-day-old seedlings urease tests showed that the enzyme was most concentrated in the elongated narrow cells of the outer cortex (pH 4.0); the inner cortex and pith (pH 5.5) contained slightly less enzyme. It was difficult to judge the urease content in lateral root primordia because of the relatively high enzyme activity of the parenchyma cells of

the collet. The lateral root primordia arising endogenously from the pericycle cells opposite the protoxylem always contained less urease than did the parenchyma cells. By removing the larger lateral roots (1 to 3 cm.) and examining them separately, it was found that they contained very little enzyme except in the apical meristems. In 4-week-old seedlings, sections of the collet indicated a low urease activity, the pith containing somewhat more urease than the cortex. In 7-week-old plants the pith gave a barely perceptible test for urease; the cortex gave no test.

Root

In the 5-cm. radicle, a distinction between root and hypocotyl is already evident, owing to the appearance of a lateral tuft of roots at the collet region. An examination was made of the urease content at different levels of the primary root. The enzyme was present in the apical meristem. It was absent in the cambium and its derivatives of secondary phloem and xylem which were already differentiated in the region 2 cm. above the tip. The

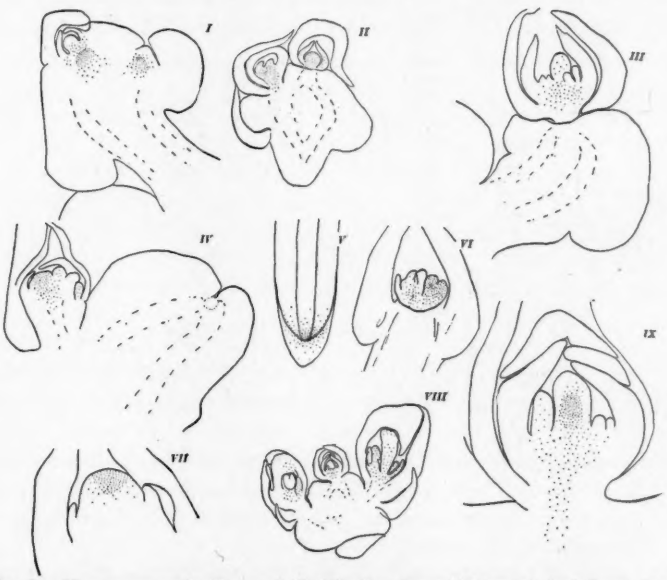

FIG. 3. Urease concentration and distribution in meristems of jack bean (as shown by intensity of stippling in camera lucida drawings).

I and II, sections made through different levels of the same pedicellar gland, showing urease in the very young flower meristems and in the gland.

III, IV, VIII, IX, urease distribution in later stages of flower differentiation.

V, distribution of urease in a longitudinal section of root tip.

VI, urease content of a cluster of promeristems of a leaf axil.

VII, distribution of urease in an apical vegetative meristem.

parenchyma cells of the cortex and pith, however, contained urease throughout the entire length of the root.

Seven days after planting, the root had increased to 20 cm. At this stage, the root tip contained very small quantities of urease. Roots, sectioned longitudinally in paraffin or on a freezing microtome, showed a positive reaction for urease in only about 5 per cent. of the root tips examined. The enzyme in these root tips was strictly delimited to the meristematic region (fig. 3, V). Whole root tips also were examined. The following modified procedure gave good results. A number of root tips were placed on a slide and treated with haematoxylin, in 20 per cent. alcohol, for 15 minutes. The dye penetrated into the cells and the root tips became yellow. (A few root tips, which appeared to be injured or sickly, turned red, that is, they were more alkaline than the rest. These were discarded.) Excess dye was drained off, 1 per cent. urea solution was added, and a cover slip applied. After 2 to 10 minutes, a red color appeared which varied from a very weak positive to a weak positive test. Every root showed this red region at the meristematic zone, and not far above it. The variations in urease distribution in the root tips examined by this method are shown in figure 4. The maximum urease

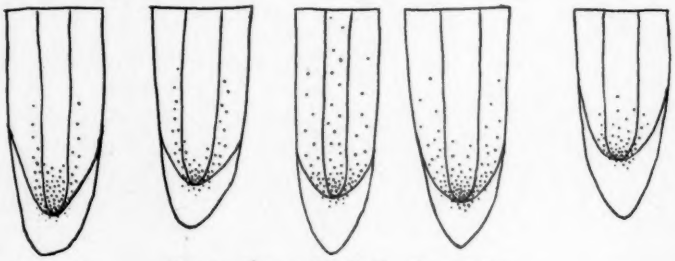

FIG. 4. Urease distribution in root tips.

content is in the region of actively dividing cells. In some root tips there is more urease in the periblem than in the plerome, and in others the opposite is true.

A large number of root tips from 13-week-old plants were examined. Most of these root tips gave a negative reaction for urease with the staining reagents. However, those that were positive showed that urease was located only in the meristematic region.

The studies of LINDERSTRØM-LANG and HOLTER (3), on the quantitative distribution of peptidase in the germinating barley root, led them to conclude that the maximum peptidase activity was related to the region of cell elongation and not to the region of rapid cell division. The experiments on urease relate the maximum urease concentration to the region of rapid cell division.

The data for the quantitative urease determinations on the roots of the jack bean are given in table II. It is seen from these figures that the urease

TABLE II

UREASE CONTENT OF ROOTS OF JACK BEAN SEEDLINGS

DAYS AFTER PLANTING	STRUCTURE	FRESH WT.	U.U. PER GM. FRESH WT.		U.U. PER UNIT STRUCTURE	
			+ COLLET	− COLLET	+ COLLET	− COLLET
days		*gm.*				
1	radicle	0.025	19.2	.....	0.480	.....
3	radicle	0.300	2.94	.....	0.824	.....
5	root + collet	0.310	0.434	.....	0.134	.....
7	root + collet	0.788	0.169	.....	0.133	.....
10	root + collet	0.645	0.191	.....	0.123	.....
20	root	0.625	.....	0.0187	.....	0.0117
.....	root + collet	0.997	0.0515	.....	0.0514	.....
28	root	2.37	.....	0.0114	.....	0.0271
40	root	3.31	.....	0.0079	.....	0.0263
.....	root + collet	3.77	0.0105	.....	0.0396	.....

activity per gram of fresh weight decreases with increasing age of the roots. This is to be expected if one assumes: that (a) the cells of the root, back of the region of cell division, do not possess any marked ability to synthesize or elaborate urease, in which case only a dilution of the enzyme already present in the cell would occur during cell elongation; that (b) owing to the partial sloughing off of the outer cortical cells of the root, and a partial disintegration of the pith, these urease-containing cells are destroyed and the enzyme in them is likewise destroyed; that (c) owing to the increase of dead xylem cells, the percentage of living cells in the root decreases and therefore the urease activity per unit of fresh weight also decreases. From the figures on the urease activity per unit tissue of "root + collet," it is seen that a definite decrease of urease must occur even in the early stages of root formation. This decrease of the enzyme is not readily evident during the first 10 days after germination because of the rapid increase in the number of new roots, and consequently of young cells containing urease. After this time, the number of roots increases slowly and the value for total urease drops off rapidly. The data also indicate that during the first 10 days the collet region possesses approximately five times as much urease activity as the roots.

HYPOCOTYL

The hypocotyl of the 5-day-old seedling possesses a high urease content throughout its entire length of 4 cm. The enzyme appears mainly in the parenchyma cells of the cortex and pith. The epidermis and the small subepidermal parenchyma cells contain little of the enzyme. No urease could be detected, with the histological reagents, in the cambium or its derivatives.

The decrease of urease activity follows the elongation of the hypocotyl as would be expected if dilution of the enzyme were taking place. The stippling in figures 1 and 5 indicates the relative urease activity of the 10-, 20-, 28-, and 40-day-old seedlings. The enzyme does not continue to decrease indefinitely, for in the 12-week-old plant the hypocotyl still gives a weak reaction in the pith and cortex with the histological reagent. The content of urease in the hypocotyl is always highest just below the cotyledons and just above the collet region, disappearing most slowly from the collet region. Whether the cells in these regions remain small, or whether less urease is catabolized, has not been determined.

From the quantitative data in table III, it may be observed that the change in urease activity is quite marked, decreasing from 0.777 U.U. per

TABLE III

UREASE CONTENT OF HYPOCOTYL (FIRST INTERNODE)

DAYS AFTER PLANTING	STRUCTURE	FRESH WT.	U.U. PER GM. FRESH WT.	U.U. PER UNIT STRUCTURE	
				ENTIRE HYPOCOTYL	PARTS OF HYPOCOTYL
days		*gm.*			
1	Radicle	0.025	19.2	0.480	.....
3	Radicle	0.300	2.94	0.824	.....
5	Hypocotyl 3.5 cm.	0.480	1.62	0.777	.....
10	Hypocotyl 7 cm.	0.921	0.561	0.518	.....
	Upper half	0.460	0.678	.....	0.312
	Lower half	0.461	0.446	.....	0.206
20	Hypocotyl 12 cm.	1.802	0.153	0.275	.....
	Upper half	0.857	0.151	.....	0.129
	Lower half	0.945	0.154	.....	0.148
28	Hypocotyl	1.401	0.0550	0.0770	.....
40	Hypocotyl	1.708	0.0317	0.0541	.....

hypocotyl in the 5-day-old seedling to 0.0541 U.U. in the 40-day-old plant. The changes in structure of the hypocotyl with age, such as the conversion of some parenchyma cells into pericyclic and collenchymatous tissue, etc., do not account for the marked decrease in total urease content of the hypocotyl. A decrease in the enzyme content of the parenchyma cells is evident.

STEM

The apex of the stem was made the subject of a detailed study since this material contains, within the short space of 3 mm., the embryonic meristem and the differentiating tissues. Longitudinal sections, cut at 35 and 60 μ were tested with the urease reagents. These showed that urease was located in greatest quantity in the primordial meristem. With the enlargement of the isodiametric cells of the meristem to form cortex and pith, the enzyme rapidly decreased in concentration, being absent in some stem tips at the region where metaxylem elements are already visible.

A few photomicrographs (fig. 6) were taken of sections of apical buds treated with the haematoxylin-nickel reagent for urease. In the presence of ammonia, a dark purple lake is formed which shows up black in the photographs. The photographs do not indicate the actual sharpness with which the enzyme may be localized by this method, since it was necessary to permit the development of a more intense and therefore a somewhat more diffuse color in order properly to photograph the sections. Figure 6 A shows two meristems containing much urease, while figure 6 B shows two meristems one of which contains more enzyme than the other.

There were great variations in the enzyme content of the various apical and axillary buds examined. Sections of some buds gave no urease test. Most of them gave only a weak reaction (fig. 3, VII). A few gave fairly strong reactions. The weakly reacting sections evidenced limitation of urease to the promeristem of the tip and to the promeristems in the leaf axils. With sections of higher urease activity both the promeristems and the procambium possessed a relatively high urease content; furthermore, the cortex, the pith, or both together, gave a positive urease reaction extending well into the elongation-region (3 mm. from the tip). The procambium strands gave weaker reactions as they differentiated, and by the time a few metaxylem elements had appeared they gave no reaction for urease. In these buds it was difficult to judge the urease content of the cambium or its derivatives because of the urease activity of the neighboring parenchyma cells. In cross-sections of young stem tissue 15 mm. below the apical bud, the enzyme was just faintly detectable only in the pith.

A comparison was made between the urease activity of the axillary and the apical buds taken from the same plant. It was found that some axillary buds contained more urease than the apical buds, or *vice versa*. It also appeared that apical buds of vigorously growing shoots possessed a somewhat higher urease activity than apical buds of slower-growing shoots.

The quantitative data on the urease content of the stem internodes (table IV) indicate that the U.U. per gram of fresh weight values are highest in the uppermost, and therefore the youngest, internodes. This is to be expected

TABLE IV

UREASE CONTENT OF DIFFERENT INTERNODES OF THE STEM

INTERNODE	AVERAGE LENGTH	FRESH WT.	U.U. PER GM. FRESH WT.	U.U. PER INTERNODE
	cm.	*gm.*		
6	3 (leaf bud)	0.0088	0.066	0.0006
5	5	0.150	0.0127	0.0019
4	10	0.585	0.0059	0.0034
3	9	0.644	0.0049	0.0028

since the histological studies on the stem tip showed that the youngest cells possessed the highest urease activity. The urease activity per gram of fresh weight in the lower internodes decreases progressively with age owing to the dilution of the enzyme and probably also to a catabolism of the enzyme.

Epicotyl

The region above the cotyledonary node, *i.e.*, the epicotyl, is considered separately from the other internodes because it is one of the organs which is already developed in the seed and these organs have been found to possess very high urease contents. Sections of the young, second internode show

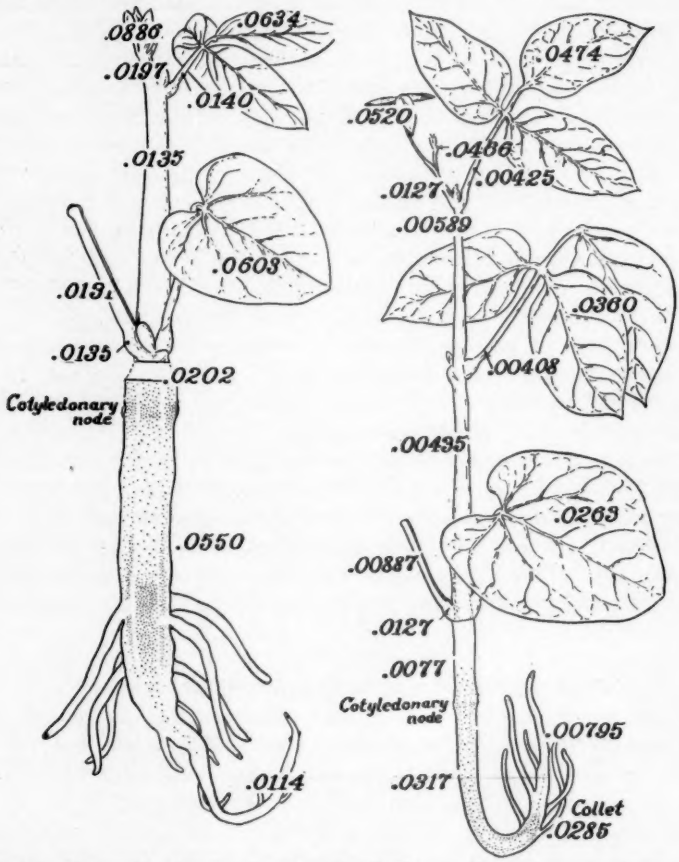

Fig. 5. Composite picture of urease distribution in the various structures of the jack bean seedlings 28 and 40 days old. (See legend of fig. 1.)

active urease in the pith and cortex. As the internode increases in length, the enzyme concentration decreases until after 28 days (fig. 5) it is detectable only close to the cotyledonary node. From the quantitative data in table V it may be seen that the urease content per epicotyl is highest when the plant is 15 days old. The most rapid elongation has occurred between the tenth and fifteenth day. After this period of rapid elongation the urease

TABLE V

UREASE CONTENT OF EPICOTYL (SECOND INTERNODE)

AGE OF PLANT	LENGTH	FRESH WT.	U.U. PER GM. FRESH WT.	U.U. PER EPICOTYL
days	*cm.*	*gm.*		
3	0.9 (plumule)	0.0120	1.35	0.0162
15	3.5	0.180	0.222	0.0401
20	5.0	0.412	0.0391	0.0161
28	5.5	0.512	0.0202	0.0103
40	5.2	0.675	0.0077	0.0052

content per epicotyl drops off markedly. Urease activity per gram of fresh weight is greatest in the meristematic tissue of the young 9-mm. plumule and decreases rapidly as these cells elongate and age. This same sequence of urease changes with age has also been found in the other stem internodes.

LEAVES

Examination of longitudinal sections of the differentiating leaves of a large number of axillary and apical buds revealed that the very young leaves enveloping the growing point contain urease, at times more, and at other times less than the growing point itself. The enzyme content of the buds as a whole varied greatly, some containing much, others little urease. No reason has as yet been found for this variation. The procambial tissue at the base of the developing leaf gave a strong reaction for urease. Even at a slightly later stage, when a portion of the procambium strands had differentiated into definite protoxylem elements, the content of urease was still high in the protophloem and procambium. No differences in urease activity were noted among the various parenchyma cells.

In a study of leaflets 10 mm. long it was found that the veins gave a stronger urease reaction than did the other portions of the leaf blade. Examination of the veins indicated that the enzyme was limited to the large area of parenchyma cells below the vascular bundles, and to a lesser degree to the bundle sheath, the reaction of the phloem being doubtful.

Sections of leaflets 4 cm. long from the first leaf below the apical bud again showed that the cortical cells of the midrib and of the other main veins contained more urease than did the other cells of the leaf blade. The strong-

est urease reaction of the leaf was given by the cortical cells of the midrib at the base of the leaf. No difference could be noted in the urease content of parenchyma cells throughout the length of the leaf. In cross-sections, the enzyme appeared to be more concentrated in the palisade cells than in the

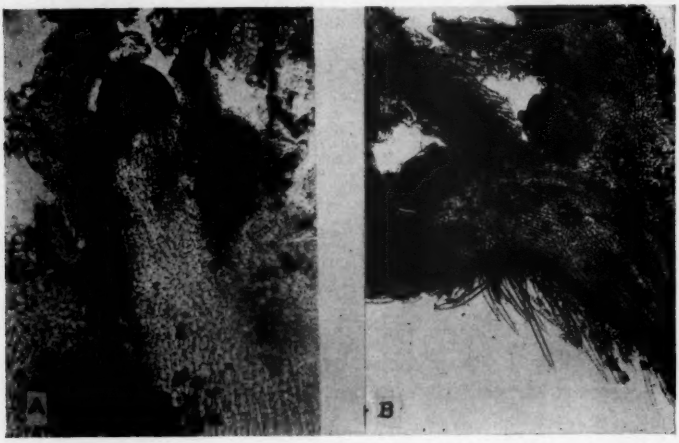

FIG. 6. Urease distribution in meristems of apical buds of jack bean. Darker areas indicate higher urease content.

spongy mesophyll. This may be due, however, to the compactness of the palisade cells which, under the conditions of the test, would effect a more rapid increase in alkalinity than would the loose mesophyll cells, even though the urease contents were the same, cell for cell. The epidermal cells appeared to contain less urease than the mesophyll cells. In leaflets 12 cm. long, no indications of the enzyme distribution could be obtained because of the low urease content.

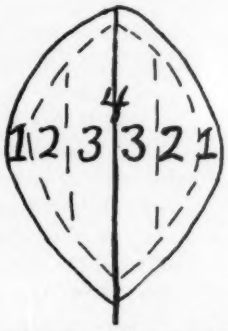

FIG. 7. Leaflet showing place from which samples were taken for urease determinations.

Quantitative determinations were made on the distribution of urease in different portions of leaflets 12 cm. long and 5.3 cm. wide. Portions of these nearly mature leaflets were taken as shown in figure 7. It is seen from table VI that the data on U.U. per gram of fresh weight indicate that urease

TABLE VI

UREASE CONTENT OF PORTIONS OF LEAFLET (FIG. 4)

No.	PART OF LEAFLET	FRESH WT.	U.U. PER GM. FRESH WT.
		gm.	
1	Outermost 5-mm. edge	0.720	0.0310
2	10-mm. middle strip	0.998	0.0345
3	10-mm. inner strip	1.161	0.0342
4	Midrib	0.365	0.0123

activity is about the same throughout the various portions of the lamina. The enzyme content of the outermost edge of the lamina is somewhat less than that of the other portions of the lamina. This may or may not be due to the fewer parenchyma cells as compared to epidermal cells in this portion of the lamina. The midrib of the leaflet contains only about one-third as much urease per gram of fresh weight as the other parts. As has been mentioned above, the parenchyma cells in the veins contain much urease. The relatively low figure for U.U. per gram of fresh weight in the midrib is due to the large proportion of primary xylem and of collenchyma cells which contain little or no urease.

A quantitative study was made on the amounts of enzyme in leaves of different sizes. Under similar environmental conditions the young, actively growing ternate leaves of the same size showed remarkably similar values for urease content. Apparently the position of the leaf on the stem makes little difference. Indeed, it was possible to predict approximately the total urease content of any size of actively growing leaf because of this constancy of relation. From table VII it is evident that the youngest tissues possess the highest urease activity per gram of fresh weight. As the leaves grow older, these values decline. The figures for "U.U. per unit structure" show clearly that a definite synthesis of urease has taken place during the expansion of the cells, and long after cell division has ceased in the young leaf. Contrasting the leaf 6.5 cm. long and the fully expanded leaf 15 cm. long, it is seen that the urease content has increased from 0.037 to 0.149 U.U. per leaf.

PLUMULE LEAVES

The first leaves (plumule leaves) to appear after the germination of the seed are a pair of simple, opposite, cordate leaves located at the second node. These were laid down in their embryonic condition within the developing

seed. As noted previously, the tissues formed in the developing seed possess, in their early stages, a higher urease activity than those which develop from the meristems after germination. Because of this higher content, detailed studies of urease distribution in these leaves could be made with the histological reagents.

TABLE VII

UREASE CONTENT OF DEVELOPING TERNATE LEAVES

STRUCTURE	FRESH WT.	U.U. PER GM. FRESH WT.	U.U. PER UNIT STRUCTURE
	gm.		
1-mm. apical bud	0.0016	0.08	0.0001
3-mm. apical bud	0.0028	0.05	0.0001
17-mm. apical bud at 6th node	0.0265	0.046	0.0012
3.5-mm. leaf	0.0088	0.066	0.0006
1.4-cm. leaf at 5th node	0.0385	0.053	0.0020
6.5-cm. leaf blade at 4th node	0.780	0.047	0.0370
15.0-cm. leaf blade at 3d node	4.115	0.036	0.149
Petiole of leaf at 4th node	0.603	0.0042	0.0026
Petiole of leaf at 3d node	0.579	0.0041	0.0024

Sections of the young plumule leaf (3 mm. long) showed a strong urease reaction throughout the leaf, the veins containing slightly less enzyme than the embryonic parenchyma cells. In the slightly older leaf (8 mm. long) the large cortical cells of the midrib contained less urease than did the embryonic parenchyma and epidermal cells. In the leaves 20 mm. long, the picture of urease distribution was similar to that obtained with the trifoliate leaves, that is, the palisade cells gave the strongest urease reaction, the mesophyll cells less, and the epidermal cells still less. Epidermal layers, stripped from the leaves and examined for urease, gave indications of the presence of only traces of enzyme. Sections through a leaf 8 cm. long from a 17-day-old plant showed that the greatest urease concentration was present in the parenchyma cells of the midrib lying below the vascular bundles. The urease reaction was especially strong at the base of the leaf, *i.e.*, in the secondary pulvinus. In the petiole proper, only a slight urease activity was noted. At the base of the petiole, in the pulvinus, only the lower portion of the cortex contained the enzyme (fig. 5).

Determinations were also made of the quantity of urease in the plumule leaves at various stages of their development. The leaves follow the same trends of urease content as those noted for the stem internodes and the roots (table VIII). The youngest leaves have the highest urease activity per gram of fresh weight and as the leaves become older the urease activity per gram of fresh weight diminishes rapidly. The urease content per unit of organ shows that a definite synthesis of the enzyme takes place until elongation of

TABLE VIII

UREASE CONTENT OF THE PAIR OF PLUMULE LEAVES

AGE OF PLANT	STRUCTURES	FRESH WT.	U.U. PER GM. FRESH WT.	U.U. PER UNIT STRUCTURE
days		*gm.*		
10	2-cm. leaves	0.0101	0.816	0.083
20	8.5-cm. leaf blades	2.066	0.0556	0.115
28	13-cm. leaf blades	4.990	0.0603	0.306
40	13-cm. leaf blades	5.730	0.0263	0.150
28	13-cm. leaf blades:			
	main veins	0.850	0.0377	0.032
	blade minus main veins	4.14	0.0662	0.274
20	Both petioles	0.538	0.0204	0.0110
28	Both petioles	0.662	0.0132	0.0088
40	Both petioles	0.783	0.0098	0.0078

the cells has practicaly ceased. From that time on inactivation or catabolism of urease occurs. The petioles undergo these changes more rapidly than do the leaves. The urease content of the plumule leaves in their adolescent stage (approximately 2 cm. long) is much higher (0.81 U.U. per gm. fresh weight) than that of the ternate leaves of similar size (0.05 U.U. per gm. fresh weight). Stipules of the second node were also examined, and, as would be expected, they undergo changes in urease content similar to the leaves.

FLOWER AND FRUIT

The flowers arise on knoblike protuberances, the pedicellar glands, which may be borne sessile in the axil of a leaf in close proximity to the axillary vegetative bud; or they may arise from a stalk, the peduncle, which may grow out to a length of 10 to 15 cm. and upon this peduncle may be borne one or more pedicellar glands, each of which may bear 3 to 5 flowers (fig. 3, IV, VIII).

The distribution of urease was studied in sections of flower primordia, in a manner similar to that described for the vegetative primordia. A photomicrograph of a longitudinal section, through a nodal region, stained with the lake reagent for urease, is shown in figure 8. It will be noted that there are several promeristems present in a very early stage of differentiation, all of which stain darker than the neighboring tissues, and thus contain more urease. It is probable that the cluster of three promeristems at the left are the "anlage" of the flower primordia and that the promeristem at the right will differentiate into a vegetative primordium. Another view of a cluster of promeristems in a leaf axil may be seen in figure 3, VI, where urease concentration is indicated by the intensity of stippling.

Urease distribution in later stages of flower differentiation is illustrated in figure 3. Figure 3, VIII shows a section through a pedicellar gland bear-

FIG. 8. Longitudinal section of axillary meristems showing the distribution of urease.

ing three flower primordia. Figure 3, I and II, are of sections made through different levels of the same pedicellar gland. They indicate that a relatively high urease activity extends from the flower promeristems into the regions of the pedicellar gland where the cells are still small and active. Later stages of flower differentiation are represented by figure 3, IV, III, and IX. From the numerous observations made on flower primordia, it may be said that, in general, urease is relatively highest in concentration in the flower promeristems; in the differentiating flower it is highest in the primordia of the pistil and the cells connecting the pistil with the pedicellar gland; it is less in the stamen primordia, and least in the primordia of the calyx and corolla.

No urease can be detected in the peduncles with the indicator reagents. A quantitative determination on peduncles varying from 1 to 9 cm. in length gave an average urease activity of 0.0165 U.U. per gram of fresh weight (table IX).

The pedicellar glands are made up of a spongy mass of parenchyma cells. Only those regions of the parenchyma which join or abut on the flower primordia give a faintly positive urease reaction with the histological re-

TABLE IX

UREASE CONTENT OF FLOWER BUDS

STRUCTURES	FRESH WT.	U.U. PER GM. FRESH WT.	U.U. PER UNIT STRUCTURE
Peduncles (1–9 cm. long)	.....	0.0165	.....
Pedicellar glands	.....	0.0199	.....
Tip of raceme; 12 flower buds each less than 2 mm. (av. wt. 0.0064 gm.)	0.0765	0.0542	0.00034 (average)
5-mm. flower bud	0.0144	0.0241	0.00035
13-mm. flower bud	0.152	0.00745	0.00112
14-mm. flower bud (just opening)	0.210	0.00683	0.00143

agents. The total urease activity of these glands is relatively low (0.0199 U.U. per gram fresh weight). From the pedicellar glands arise the flower primordia which contain a fair amount of urease (0.0542 U.U. per gm. fresh weight). As growth and differentiation of these cells continue, urease activity decreases (table IX), until, in the flower bud which is just opening, it is down to 0.0068 U.U. per gram of fresh weight. Although the urease activity per gram of fresh weight has decreased, there has been a definite synthesis of urease per flower bud.

When the flower is mature the pollen grains possess a low but definite urease activity. The pistil at this stage also has a low urease activity (0.0028 U.U. per gm. fresh weight).

Since the endosperm contains 3n or 33 chromosomes, it was considered especially interesting to determine the urease activity of this tissue. Sections of a bean 10 mm. long treated with a urease reagent showed that urease, if present in the endosperm, was below that concentration which could be detected by the histological method. It was however found that the endosperm could be readily removed *in toto* from the embryo sac. A number of endosperms were therefore removed and placed in small test-tubes and urea and indicator added until the total volume was 0.20 cc. Appropriate controls were also run. The endosperm tissue was not crushed. After one hour a definite positive indication of the presence of urease in the endosperm was observed. It is estimated that the endosperm had an approximate urease activity of 0.005 U.U. per gram of fresh weight. This same procedure was used in determining the presence of urease in mature pollen grains.

In a seed, 10 mm. long, the radicle of the embryo is somewhat less than 1 mm. The radicle at this stage possesses a definite but low urease activity. The cotyledons, consisting of small thin-walled cells, also possess a low urease activity. The inner integument consists of two layers of parenchyma cells and an epidermal layer abutting on the embryo sac. This integument has the highest urease activity of any of the tissues in a seed 10 mm. long, the urease activity being somewhat greater at the chalazal end.

The quantitative data (table X) on the urease content of the maturing bean reveal a steady increase of urease as the bean develops. In the young bean the weight of cotyledon and endosperm is negligible compared to that of the testas, and the values for U.U. per gram of fresh weight and for U.U. per bean up to the stage where the bean is 13 mm. long actually indicate the changes occurring in the testas. The testas pass through the same stages as other structures previously described. There is a decrease of U.U. per gram of fresh weight as the cells of the testa enlarge and mature; however, the urease activity per testa increases until the bean is approximately 15 mm. long and thenceforth decreases as the bean continues to enlarge.

TABLE X

UREASE CONTENT OF THE MATURING BEAN

LENGTH OF BEAN	FRESH WT.	U.U. PER GM. FRESH WT.	U.U. PER UNIT STRUCTURE
	gm.		
4– 6-mm. bean	0.0117	0.0822	0.00096
7– 9-mm. bean	0.0575	0.0467	0.0027
11–13-mm. bean	0.185	0.0262	0.0048
17-mm. bean	0.648	.....	0.178
Embryo	0.352	0.495	0.174
Testa	0.296	0.0162	0.0048
20-mm. bean	1.080	.....	0.400
Embryo	0.646	0.615	0.397
Testa	0.434	0.0080	0.0035
22-mm. bean minus testa	1.60	.....	2.65
Cotyledons	1.58	1.65	2.61
Radicle and plumule	0.0162	2.75	0.044

As the cells of the cotyedons continue to divide and enlarge in the developing embryo, storage of proteins, starch, and hemicelluloses proceeds vigorously and the urease activity likewise increases very rapidly until the bean has attained its full size. In the radicle and plumule the cells elongate only slightly; there is less storage of carbohydrates than in the cotyledons but the cells accumulate proteins and also increase rapidly in urease activity. From the data in table X it is seen that the urease activity of the radicle and plumule of the mature bean (2.75 U.U. per gm. fresh weight) is even higher than the urease activity of the cotyledons (1.65 U.U. per gm. fresh weight). This dominant synthesis of proteins together with urease in meristematic cells of the embryo has already been noted for the other meristematic tissues of the plant with the exception of the cambium, where no detectable amount of urease is produced.

ENTIRE PLANT

A survey of the changes in the urease content of the jack bean plant as a whole, throughout its life cycle, is given in the graph of figure 9. Total

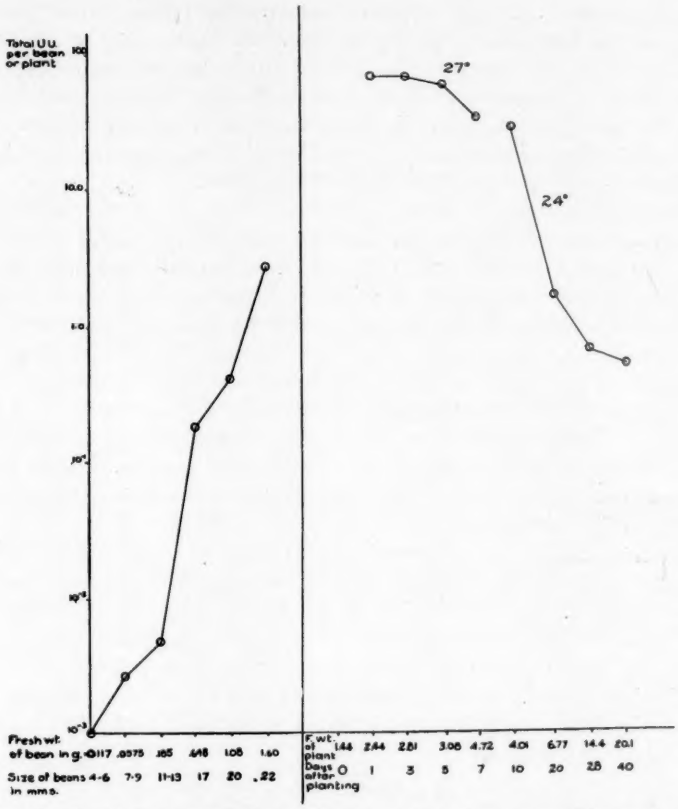

FIG. 9. Graph representing the total urease content of young maturing seeds and of seedlings at various ages after planting. "Total U.U. per bean or plant" is plotted logarithmically along the axis of ordinates. Various stages of seed or plant development are plotted on the axis of abscissas.

urease is plotted logarithmically on the ordinate, and the various stages of development are indicated on the abscissa. As the beans develop and mature in the pods, a rapid synthesis of urease takes place, the urease values increasing over 1000 times when a comparison is made between the urease content of the very young bean (4–6 mm. long) and the nearly mature bean (22 mm. long). The stages of complete maturity and drying were not determined in this plant.

Upon germination the seedlings develop rapidly. Thermograph records were kept and the average temperatures noted at which urease in the seedlings decreases with age, changing from 66 U.U. per seedling after one day's ger-

mination to 0.45 U.U. in the 40-day-old plant. The most rapid decrease in urease content occurred in the 10- to 20-day-old plant. In the 20-day-old plant the cotyledons were about to fall off. Although total urease determinations were not carried out on plants over 40 days of age, it may be said from other data that there will be a slight increase in urease content when more leaves have developed on the plant. Still later, when the seeds begin to form in the pods, a marked increase will take place.

When the data on the urease content of the other plant structures are considered, it becomes evident that figure 9 represents, to a large degree, the urease changes of the cotyledons. The cotyledons contain such preponderating quantities of urease as to overshadow any changes of urease content occurring in the other plant structures. Even when the cotyledons are about to fall off in the 20-day-old plant, they still contain about two-thirds of the total urease of the plant. The marked decrease in urease in the 10- to 20-day-old plant is due to the marked decrease in urease content of the cotyledons. It is interesting to note that even after the cotyledons have fallen off, the urease content per plant still continues to decrease. The decrease in the total amount of urease in the plant is therefore not wholly due to the decrease in the cotyledons.

The distribution of the enzyme in the various tissues of seedlings 10, 20, 28, and 40 days old is illustrated in composite pictures in figures 1 and 5. The stippling in these figures indicates the relative urease concentrations as revealed by histological methods. The figures opposite each structure express the urease units per gram of fresh weight as determined by the quantitative method. A description of the specific urease changes of the individual plant structures will be found under their respective headings in the previous pages.

Discussion

It was realized, only after the analyses were nearing completion, that the urease content in different plant structures, as the internodes, leaves, roots etc., follow similar trends which are related to the ages of these structures. The curves in figure 10, in which the relative ages of the structures are plotted along the abscissa, indicate these general trends. The urease activity per gram of fresh weight (fig. 10 A) is highest when the cells of the young structure are actively dividing, and decreases as the cell of the primary tissues[4] grow older. The total urease content of a plant region (fig. 10 B) increases rapidly during the period of cell division of the primary tissues, and more slowly during the period of cell elongation until, at about the time

[4] The term primary tissues, as here used, has reference to those tissues developing from the apical meristems in contrast to the secondary tissues which develop from the cambium.

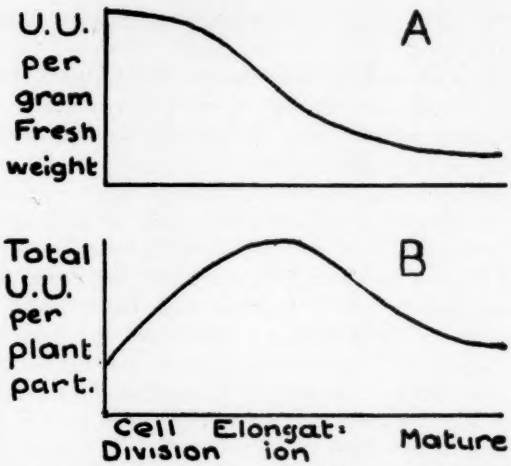

FIG. 10. Changes in urease activity in relation to physiological age.

primary growth has ceased, the plant part contains a maximum urease content.

Consideration of the histology of the seedlings makes it evident that the preponderating cells are the parenchyma cells. These cells originate by the elongation of the isodiametric cells of the apical meristems and appear to be the least differentiated of the cells derived from the meristems. Since it has been found, with the indicator reagents, that neither the cambium nor its derivatives contain perceptible quantities of urease, it becomes apparent that the changes observed in the distribution of urease are really the changes occurring in a single tissue, the parenchyma.

Upon this basis, the urease changes may be related to the aging of parenchyma cells. The urease content per unit volume of cell steadily decreases (fig. 7 A) as a cell ages. The decrease in urease units per gram of fresh weight is especially rapid during the period of cell elongation. After that, there is a slower decrease in the enzyme. This decrease is partially accounted for by the dilution of materials in the cell and by an increase in dry weight due to cellulose deposition, but there is also a definite inactivation or perhaps catabolism of urease taking place especially in the mature cells.

The metabolism of urease in the parenchyma cells may be briefly summarized (fig. 10 B) thus:

1. There is a rapid synthesis of urease in the actively dividing meristematic cells.

2. This synthesis continues during the period of cell elongation although at a decreasing rate, the amount of urease of a cell reaching a maximum at the end of the elongation period.

3. After this period, there is a decomposition of urease which continues down to a certain level.

A discussion of reasons for these changes will appear in the next paper of this series. The parenchyma cells of the root pass through the various growth stages and phases of urease change most rapidly, the stem cells less rapidly and the leaf cells least rapidly.

The cells possessing the densest cytoplasm contain the highest content of urease. This is true for the meristems of the root tip, stem apex, internodes, and flower promeristems. In the cotyledons the subepidermal parenchyma cells, the cells making up the bundle sheaths of the veins, and the young primary phloem cells, which appear to contain the densest cytoplasm, are also richest in urease. The density of cytoplasm is related to its protein content (the major portion of the protein being globulin). If the globulin urease undergoes changes similar to other globulins in synthesis and decomposition, it might be expected that an increase in protein content of the cytoplasm would be accompanied by an increase in urease activity. The data appear to confirm this.

The structures developing from the fertilized egg and laid down in the maturing seed have been treated separately from the structures which are differentiated after the seed germinates because the former possess a urease activity which is higher than that of similar organs developing in the seedling or mature plant. For example, at comparable stages of growth the plumule leaves (2 cm. long) have a urease activity per gram of fresh weight of over 15 times that of the ternate leaves of the same size; likewise, the young epicotyl (second internode) has a urease activity per gram of fresh weight of over 20 times that of an upper internode of similar weight. The cause of this difference in urease activity may be traced back to the development of the maturing seed, during which period there is a marked protein synthesis and storge including urease synthesis and storage. Upon germination, the meristematic cells of the radicle and plumule not only start with a high content of urease but are supplied by the cotyledons with a large amount of nitrogenous compounds from which to synthesize more urease during cell division and cell elongation. The later-developing structures are poorer in urease content, which is probably due to the fact that they are less richly supplied with nitrogenous compounds and must depend for their nitrogen on the mature and senescent cells and upon the supply of inorganic nitrogen obtained from the soil.

Summary

1. The urease activity of *Canavalia ensiformis* has been determined using histological and quantitative methods. The data for total urease are summarized in figure 6 which is a graph showing the total urease content of the developing bean, and of the young plant through the 40-day-old stage. The distribution of urease in the various tissues of seedlings 10, 20, 28, and 40

days old is illustrated in a composite picture (figs. 1, 5). Stippling indicates the relative urease concentrations as revealed by the histological methods. The figures opposite each plant part express the urease activity in terms of urease units per gram of fresh weight as determined by the quantitative method. A description of the specific urease changes in the individual plant structures is given under their respective headings in the previous pages.

2. The changes observed in the urease content of the plant are due primarily to the changes in urease content of the parenchyma cells. Neither the cambium nor the cells derived from the cambium, that is, the phloem and xylem, contain any amounts of urease that can be detected with the indicator reagents.

3. The changes of urease activity in the parenchyma cells of any organ follow the same trends with aging of the cell. There is a rapid synthesis of urease in actively dividing cells. This synthesis continues during the elongation stage of the cell, the urease content of a cell reaching a maximum at the end of the elongation stage. After this stage, there is a decrease of the enzyme which continues until the urease content of the cell is down to a certain level.

4. The cells of the embryo developing from the fertilized egg and laid down in the maturing seed possess a urease activity which is higher than that of cells in similar organs developing in the seedling or mature plant.

The writer desires to express his gratitude to Professor F. G. GUSTAFSON for the many helpful suggestions and valuable criticisms throughout the course of this investigation and to Mr. HARMAN DUNHAM for assistance in taking the photomicrographs and in the preparation of the illustrations.

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NUTRITIONAL CHARACTERISTICS OF CERTAIN WOOD-DESTROYING FUNGI, *POLYPORUS BETULINUS* FR., *FOMES PINICOLA* (FR.) COOKE, AND *POLYSTICTUS VERSICOLOR* FR.

H. H. LaFuze
(WITH TWO FIGURES)

Introduction

Wood-destroying fungi vary greatly in their ability to attack different kinds of woods under natural conditions. A large number of them, such as *Polystictus versicolor* Fr., grow on a wide range of tree species. Many, however, are restricted to growth on a limited number of hosts, *e.g., Polyporus betulinus* Fr. attacks only birches, while *Fomes pinicola* (Fr.) Cooke is most commonly found on gymnosperms, and rarely on angiosperms. This specificity for certain substrates is obviously an inherent physiological characteristic of these fungi, conditioned by certain substances in woods. The experiments here reported are an attempt to determine some of the physiological differences between the wood-rotting forms, *Polyporus betulinus, Fomes pinicola,* and *Polystictus versicolor.* Emphasis is placed on the nutritional and growth characteristics shown by the organisms on various artificial media.

Investigations on durability of woods have suggested a number of factors which may be responsible for the selective growth of certain fungi on specific substrates. These factors, as commonly reviewed in the literature (**1, 5, 6, 8, 18**), include the presence of toxic water-soluble extractives such as phenolic compounds, alkaloids, and fatty acids, the waterproofing effect of resins, the toxicity of resins, pine oils, terpenes, quinones, and volatile materials, the specific gravity, moisture content, and air-water balance in wood, and the nutritional characteristics of woods, especially those of nitrogenous nature. Woods which have been subjected to high temperatures and long water treatments are invariably decayed to a greater extent than untreated woods (**1, 10, 18**). This loss of immunity to decay by the woods is undoubtedly caused by changes in water-soluble and volatile materials occurring in woods during water treatments and subsequent heating in an autoclave. Experiments have been included in this investigation to determine the influence of certain water-soluble substances, commonly present in woods, on the growth of the three fungi.

Methods

The strains of fungi used in these experiments were obtained from the Forest Products Laboratory of Madison, Wisconsin. They were received as

Polystictus versicolor, no. D. 1524, isolated from oak wood; *Fomes pinicola*, no. 610, isolated from a sporophore; and *Polyporus betulinus*, no. 652, isolated from sporophore tissue. Stock cultures of these were grown for two years preceding these experiments on media consisting of 1 per cent. Spramalt extract and 1.5 per cent. agar.

The basic components used in making experimental media and the exact formula of the control medium were as follows:

BASIC FORMULA, CONTROL MEDIUM

Carbohydrate, maltose	40 gm.
Nitrogen, Witte's peptone	4 gm.
Mineral salt, dipotassium acid phosphate	1 gm.
Substratum, agar-agar	15 gm.
Water, distilled water to make	1000 cc.

U.S.P. and C.P. chemicals were used in the preparation of the media, with a maximum error in weighing of 0.5 per cent. A complete series of media differing only in the quality of a single nutrient in the basic formula was prepared at one time. The different kinds of this variable nutrient were weighed into a series of small beakers. The remaining ingredients of the control medium were heated in a large beaker to melt the agar and dissolve the solutes. This was then apportioned quantitatively among the original small beakers of the series containing the variable component after which the contents were again heated and stirred thoroughly. The pH was adjusted between 5.5 and 6.0 by neutralizing with NaOH or HCl. The media were then tubed in dry test-tubes which had been thoroughly cleaned. The tubes were plugged with cotton and autoclaved at 15 pounds pressure and about 120° C. for 15 minutes. All tubes were slanted uniformly and allowed to cool.

The inoculum, consisting of a cube of substrate not exceeding eight cu. mm., was cut from six-day-old stock cultures growing on the malt extract-agar medium. Triplicate cultures for each fungus on each medium in a series were incubated in darkened cabinets at a temperature which varied between 24° and 28° C.

Data were recorded on the tenth day after inoculation. The extent of growth over the surface of the substrate was measured from the inoculum to the farthest edge of the mycelial mat and was expressed as millimeters of linear growth. This linear measurement of mycelial mats, which is commonly used as an index of growth, was considered inadequate by itself in expressing growth since it did not take into consideration the amount of mycelium produced per unit area of surface. This objection was overcome in part by allowing the arbitrary value of five to represent the compactness and thickness of the most luxuriant mycelial mats, such as those produced by *Poly-*

stictus versicolor on the control medium, and then estimating the relative thickness of the other mats in terms thereof. The ratings were made according to the following scale: 0 indicating no growth, 1 scanty, 2 thin, 3 good, 4 abundant, and 5 luxuriant growth. These values were referred to as rates of thickness of mycelium.

The woods used were *Tilia americana* L., *Betula nigra* L., *Betula papyrifera* Marsh., *Maclura pomifera* Schneid., *Quercus alba* L., *Pinus strobus* L., *Juniperus virginiana* L., *Juglans cinerea* L., and *Platanus occidentalis* L. Tree trunks about six inches in diameter were cut during June in eastern Iowa. Sawdust from each wood was ground in a pulp-mill to pass a 40-mesh sieve and stored in air-tight mason jars till used.

Aqueous extracts of the woods were made by adding about 50 gm. of the prepared sawdust to 750 cc. of distilled water, and autoclaving at 15 pounds pressure for 1.5 hours. The total volume of extract for each wood was evaporated to 450 cc. over a steam bath, thus producing an extract, 9 cc. of which represented about one gram of wood. A series of media was prepared by using the aqueous extracts of different woods instead of the water in the control medium. The formula of the control medium was further altered to include two per cent. agar. The approximate pH of each medium was determined.

Pieces of different woods measuring $1/4 \times 3/4 \times 2$ inches were cut and trimmed until they were similar with respect to relative percentage of sapwood and heartwood, absence of knots and abnormalities, and uniformity in size and shape. After soaking in distilled water for 24 hours at 21° C., like species of wood were strung in groups of four on nichrone wire and suspended over 20 cc. of water in 250-cc. wide-mouthed Erlenmeyer flasks, which were stoppered with corks containing two pieces of glass tubing plugged with cotton. These flasks were then autoclaved at 15 pounds pressure for 15 minutes, and allowed to set in the laboratory for two weeks to test their sterility. They were finally divided into two lots and inoculated with *Polystictus versicolor,* and *Polyporus betulinus,* respectively. The cultures were incubated eight months in a dark room at a temperature of 22°–25° C. and a high relative humidity.

Experimental results

Carbohydrate nutrition

Various carbohydrates were substituted for maltose in the control medium. The fungi developed the greatest amount of mycelium in the presence of inulin, followed by dextrin, maltose, levulose, glucose, mannose, starch, and raffinose (table I). The least growth was observed in the presence of glucosides, and no growth occurred on lactose.

Fomes pinicola was the most variable of all the fungi in its growth on this

TABLE I

GROWTH OF THE FUNGI ON ARTIFICIAL MEDIA CONTAINING DIFFERENT CARBOHYDRATES (4.0%). LINEAR GROWTH REPRESENTS DISTANCE FROM INOCULUM TO EXTREMITY OF MYCELIAL MAT; RATE OF THICKNESS REPRESENTS THICKNESS OF MYCELIUM IN TERMS OF AN ARBITRARY VALUE (PAGE 627). PERCENTAGES FOR EACH FUNGUS ARE IN TERMS OF THE GROWTH OF THAT SPECIES ON THE CONTROL MEDIUM*

MEDIUM	*POLYPORUS BETULINUS*				*FOMES PINICOLA*				*POLYSTICTUS VERSICOLOR*			
CARBOHYDRATE	LINEAR GROWTH		THICKNESS		LINEAR GROWTH		THICKNESS		LINEAR GROWTH		THICKNESS	
	mm.	*%*	*rate*	*%*	*mm.*	*%*	*rate*	*%*	*mm.*	*%*	*rate*	*%*
None	6	38	1	50	22	76	1	20	28	65	1	20
Arabinose	18	112	2	100	22	76	2	40	39	91	4	80
Xylose	20	125	2	100	19	66	2	40	38	88	3	60
Rhamnose	10	62	1	50	15	52	1	20	33	77	1	20
Glucose	18	112	2	100	27	93	5	100	40	93	3	60
Levulose	18	112	2	100	27	93	5	100	43	100	4	80
Galactose	18	112	2	100	23	79	1	20	40	93	4	80
Mannose	18	112	2	100	24	83	5	100	42	98	4	80
Sucrose	21	131	2	100	28	97	2	40	41	95	4	80
Maltose*	**16**	**100**	**2**	**100**	**29**	**100**	**5**	**100**	**43**	**100**	**5**	**100**
Lactose	0	0	0	0	0	0	0	0	0	0	0	0
Raffinose	19	119	2	100	25	86	4	80	42	98	5	100
Dextrin	20	125	2	100	32	110	5	100	46	107	5	100
Inulin	30	187	2	100	33	114	3	60	51	118	5	100
Corn starch	16	100	2	100	31	107	5	100	40	93	5	100
Gum arabic	14	88	1	50	27	93	1	20	38	88	4	80
Gum tragacanth	21	131	2	100	26	90	5	100	35	81	2	40
Glycerin	16	100	2	100	16	55	3	60	31	72	4	80
Saponin	0	0	0	0	1	3	2	40	21	49	5	100
Salicin	9	56	2	100	15	52	1	20	16	37	4	80
Amygdalin	2	12	3	150	4	14	1	20	3	7	4	80

* Control medium.

series of media. Pentoses and pentosans were especially poor nutrients, and the glucosides were definitely inhibitive to its growth. The most luxuriant growth was observed on glucose and its condensation products, dextrin, starch, and maltose, although good growth occurred on levulose, gum tragacanth, and mannose.

Polystictus versicolor grew relatively well in the presence of unusually high concentrations of glucosides, especially four per cent. saponin, which was quite toxic to the other wood-destroying fungi. Although the actual amount of mycelium produced by *Polyporus betulinus* on these media was very much less than that of the other fungi, it was more uniform in thickness. Contrasting with other fungi was the very extensive linear growth of the birch fungus in the presence of inulin and the complete inhibition of growth by saponin.

Nitrogen nutrition

Different nitrogen compounds were substituted for Witte's peptone in the control medium. A general survey of the data shows that the best growth of the fungi was obtained on media containing, in order of diminishing amounts, proteins, peptones, amino acids, and inorganic nitrogenous salts (table II). The best inorganic source of nitrogen was ammonium nitrate. There was only slight indication that ammonia may be better utilized than nitrate. Sodium nitrite and urea were apparently non-nutritive to all three fungi. The monamino-dicarboxylic acids, aspartic and glutamic, supported good fungal growth, while glycine, a monocarboxylic acid, was a poor nutrient. Growth was almost completely inhibited by the two amino acids, tyrosin and cystine, which contain phenyl and disulphide radicals, respectively.

Characteristic of the fungi was the luxuriant growth on gliadin, a protein which contains relatively large amounts of ammonia, tryptophane, and glutamic acid. Less abundant growth occurred on glutenin and casein, both of which are low in glutamic acid, ammonia, and tryptophane content but contain relatively more tyrosin than the other proteins. The hydrolysate of casein, deficient in tryptophane, consistently produced less mycelium than casein itself. It was found that nitrogen sources of high molecular weight, those containing tryptophane, ammonia, and glutamic acid and those low in tyrosin content favor optimum growth of the fungi.

Fomes pinicola was most tolerant to the variety of nitrogen sources supplied in this series of media. Exceptionally good growth occurred in the presence of the ammonium and nitrate salts. The organism developed better on cystine and glycine than either of the other fungi. In contrast, *Polyporus betulinus* grew poorly on this series of media. Striking variations in its growth as compared with the other fungi were noticed in the greatly increased thickness, but much reduced linear growth of mycelium, in the retardation of

TABLE II

GROWTH OF THE FUNGI ON ARTIFICIAL MEDIA CONTAINING DIFFERENT NITROGEN SOURCES (0.4%). LINEAR GROWTH REPRESENTS DISTANCE FROM INOCULUM TO EXTREMITY OF MYCELIAL MAT; RATE OF THICKNESS REPRESENTS THICKNESS OF MYCELIUM IN TERMS OF AN ARBITRARY VALUE (PAGE 627). PERCENTAGES FOR EACH FUNGUS ARE IN TERMS OF THE GROWTH OF THAT SPECIES ON THE CONTROL MEDIUM*

MEDIUM	*POLYPORUS BETULINUS*				*FOMES PINICOLA*				*POLYSTICTUS VERSICOLOR*			
NITROGEN	LINEAR GROWTH		THICKNESS		LINEAR GROWTH		THICKNESS		LINEAR GROWTH		THICKNESS	
	mm.	%	*rate*	%	*mm.*	%	*rate*	%	*mm.*	%	*rate*	%
None	25	125	1	33	24	104	1	25	43	107	1	25
NH_4NO_3	17	85	2	66	29	126	3	75	26	65	2	50
$NaNO_2$	0	0	0	0	0	0	0	0	0	0	0	0
$NaNO_3$	13	65	1	33	29	126	3	75	17	42	1	25
NH_4Cl	15	75	1	33	27	117	3	75	25	62	2	50
Glycine	2	10	5	166	19	83	2	50	43	107	1	25
Aspartic acid	20	100	2	66	37	161	3	75	42	105	2	50
Tyrosin	1	5	1	33	2	9	1	25	2	5	1	25
Glutamic acid	19	95	2	66	30	130	3	75	36	90	2	50
Cystine	3	15	3	100	15	65	4	100	24	60	2	50
Gliadin	20	100	4	133	35	152	5	125	50	125	5	125
Gelatin	16	80	2	66	29	126	3	75	40	100	3	75
Egg albumin	19	95	2	66	20	126	3	75	42	105	5	125
Witte's peptone*	**20**	**100**	**3**	**100**	**23**	**100**	**4**	**100**	**40**	**100**	**4**	**100**
Glutenin	15	75	3	100	22	96	5	123	46	115	4	100
Casein	18	90	3	100	25	108	3	75	50	125	5	125
Casein hydrolysate	11	55	3	100	20	87	2	50	45	112	3	75
Urea	0	0	0	0	0	0	0	0	0	0	0	0

* Control medium.

growth on tryptophane-deficient casein hydrolysate and gelatin, as well as on glutenin, egg albumin, and particularly cystine, and in the inhibition of growth by both sodium nitrate and ammonium chloride as compared with media lacking any nitrogen source.

Mineral nutrition

The formula of the control medium was varied by substituting different salts for the dipotassium acid phosphate at the same concentration, one gram per liter of medium. Potassium and phosphate ions were essential for good growth of the fungi (table III). The presence of potassium was correlated with a slight increase in the spread of mycelium, while that of phosphate was correlated with a conspicuous increase in thickness of mycelial mats. Of secondary importance to potassium and phosphate was calcium, which generally caused an increase in growth, particularly of *Fomes pinicola*. The presence of sodium ions appeared to retard growth especially if the spread of mycelium be taken as a criterion. This was noticeable also in the tripotassium acid phosphate medium which had been neutralized with sodium hydroxide. The medium to which no mineral salts were added supported fungal growth equal to that observed on other media containing salts, suggesting that nutritive salts might have been introduced into the first medium as an impurity of some other ingredient.

Growth of *P. betulinus* was especially scanty when compared with that of the other species. Both the birch and conifer fungi were more variable in growth responses to different mineral salts than *P. versicolor*, further emphasizing the specificity of *P. betulinus* and *F. pinicola* for certain nutrients.

Effect of pH on growth

The acidity of a short series of media made from the formula of the control medium was varied from pH 4 to 8 by adding hydrochloric acid or sodium hydroxide. The best growth of the three fungi was on an acid medium of pH 5.6, determined with the aid of a Lamotte Roulette Comparator. The three species did not differ noticeably in their pH requirements for optimum growth. Acid media tended to cause greater spread of mycelium and thinner mycelial mats than more alkaline media.

A series of media was prepared in which acidity changes of the substrate could be observed during the growth of the fungi. Different indicators were added to respective 6-cc. portions of the control medium in the following concentrations:

6 drops of 0.01 per cent. methyl orange
4 " " 0.01 per cent. Congo red
6 " " 0.02 per cent. methyl red
4 " " 0.01 per cent. neutral red

TABLE III

GROWTH OF THE FUNGI ON ARTIFICIAL MEDIA CONTAINING DIFFERENT MINERAL SALTS (0.1%). LINEAL GROWTH REPRESENTS DISTANCE FROM INOCULUM TO EXTREMITY OF MYCELIAL MAT; RATE OF THICKNESS REPRESENTS THICKNESS OF MYCELIUM IN TERMS OF AN ARBITRARY VALUE (PAGE 627). PERCENTAGES FOR EACH FUNGUS ARE IN TERMS OF THE GROWTH OF THAT SPECIES ON THE CONTROL MEDIUM*

MEDIUM	*POLYPORUS BETULINUS*				*FOMES PINICOLA*				*POLYSTICTUS VERSICOLOR*			
MINERAL SALT	LINEAR GROWTH		THICKNESS		LINEAR GROWTH		THICKNESS		LINEAR GROWTH		THICKNESS	
	mm.	*%*	*rate*	*%*	*mm.*	*%*	*rate*	*%*	*mm.*	*%*	*rate*	*%*
None	15	63	2	66	24	80	2	40	42	93	2	40
KH_2PO_4	21	88	3	100	30	100	5	100	50	111	5	100
K_2HPO_4*	**24**	**100**	**3**	**100**	**30**	**100**	**5**	**100**	**45**	**100**	**5**	**100**
K_3PO_4	3	13	5	166	19	63	5	100	35	78	5	100
Na_2HPO_4	2	8	5	166	7	23	3	60	31	68	5	100
$CaHPO_4$	15	63	3	100	24	80	4	80	40	89	5	100
KCl	13	54	3	100	26	87	2	40	43	96	2	40
NaCl	11	46	2	66	20	67	2	40	42	93	2	40
K_2SO_4	12	50	2	66	28	93	2	40	42	93	2	40
Na_2SO_4	12	50	2	66	23	77	2	40	41	91	2	40
$CaSO_4$	10	42	2	66	25	83	4	80	47	103	2	40
$CaCl_2$	11	46	2	66	21	70	3	60	43	95	2	40

* Control medium.

5 " " 0.04 per cent. bromphenol blue
4 " " 0.04 per cent. bromcresol purple
4 " " 0.04 per cent. bromthymol blue

The original pH of the medium was about 5.6. The changes in color occurring during growth of the fungi were observed under the periphery and the older portions of the mycelial mats.

There was a general tendency toward increased acidity in cultures of all three fungi. This was particularly noticed by a change in color of the indicators slightly in advance, but mostly directly beneath, the very young and actively growing mycelium. The colors due to indicators disappeared under the older mycelium. This was attributed to the alteration or destruction of the indicator by the organisms, since acids and alkalies added to the substrates caused no reappearance of the indicator color. The substrates of *Fomes pinicola* became the most acid, estimated to be about pH 3. Those of *Polyporus betulinus* were almost as acid, while the *Polystictus versicolor* substrates remained strikingly more alkaline, or about pH 4.5.

Oxidation-reduction phenomena

Oxidation and reduction indicators were added to respective portions of media prepared from the formula of the control medium. Oxidation was observed in the media growing *Polystictus versicolor* and containing guaiacol, resorcinol, and tannin, while a doubtful reaction was obtained in media containing tyrosin, and quinol (table IV). Of the other two fungi, only *F. pinicola* showed a positive reaction with any of the oxidation indicators, producing but slight coloration in the tyrosin medium.

TABLE IV

Oxidation and reduction reactions of the fungi grown on the control medium in the presence of indicators. No growth is indicated by 0, no reaction by −, weak positive reaction by +, moderate positive reaction by ++, and strong positive reaction by +++.

Medium / Indicator	*Polyporus betulinus*	*Fomes pinicola*	*Polystictus versicolor*
0.1 per cent. guaiacol	0	−	+
0.02 " " α-naphthol	0	0	0
0.1 " " tyrosin	−	+	++
0.02 " " pyrogallol	0	0	0
0.1 " " resorcinol	−	−	+++
0.1 " " tannin	−	−	+++
0.02 " " methylene blue	+++	−	−
0.002 " " methylene blue	+++	++	+

TABLE V

GROWTH OF THE FUNGI ON ARTIFICIAL MEDIA CONTAINING DIFFERENT PHENOLIC COMPOUNDS (0.02%) IN ADDITION TO NUTRIENTS OF THE CONTROL MEDIUM. LINEAR GROWTH REPRESENTS DISTANCE FROM INOCULUM TO EXTREMITY OF MYCELIAL MAT; RATE OF THICKNESS REPRESENTS THICKNESS OF MYCELIUM IN TERMS OF AN ARBITRARY VALUE (PAGE 627). PERCENTAGES FOR EACH FUNGUS ARE IN TERMS OF THE GROWTH OF THAT SPECIES ON THE CONTROL MEDIUM*

MEDIUM	*POLYPORUS BETULINUS*				*FOMES PINICOLA*				*POLYSTICTUS VERSICOLOR*			
PHENOLIC COMPOUND	LINEAR GROWTH		THICKNESS		LINEAR GROWTH		THICKNESS		LINEAR GROWTH		THICKNESS	
	mm.	*%*	*rate*	*%*	*mm.*	*%*	*rate*	*%*	*mm.*	*%*	*rate*	*%*
None*	**17**	**100**	**3**	**100**	**30**	**100**	**4**	**100**	**43**	**100**	**5**	**100**
Guaiacol	2	12	4	133	10	33	5	125	42	98	5	100
Resorcinol	16	94	3	100	27	90	3	75	34	80	5	100
Quinol	15	88	3	100	31	103	4	100	12	28	5	100
Pyrogallol	0	0	0	0	0	0	0	0	0	0	0	0
α-Naphthol	0	0	0	0	0	0	0	0	0	0	0	0
Tannin	17	100	3	100	20	66	5	125	47	108	5	100

* Control medium.

Reduction of methylene blue to the leuco-compound occurred in *F. pinicola* and *P. betulinus* cultures. *F. pinicola* was the most active in reducing this indicator. The whitened substrate soon regained a deep blue color which later changed to green, due to re-oxidation of the leuco-compound. The medium in *P. betulinus* cultures remained white for three weeks without indication of re-oxidation of the indicators. *P. versicolor* decolorized methylene blue only after cultures had been kept two weeks. The reaction, however, was limited to immediately below the mycelial mat and was almost immediately followed by an oxidation reaction, producing a green color.

Oxidase studies were continued by treating portions of the mycelium and the substrate on which the fungi had grown with a solution of guaiacum. The substrate of *Polystictus versicolor* cultures gave a strong blue color in the presence of the oxidase indicator, while the mycelium showed a weaker color reaction but nevertheless positive. Neither the mycelium nor the subtrates of *F. pinicola* and *P. betulinus* produced a positive test for oxidase.

Growth in the presence of phenolic compounds

Different phenolic compounds were added to respective portions of a medium made from the formula of the control medium. The pH was not adjusted. No growth occurred in the presence of 0.02 per cent. alpha-naphthol and pyrogallol (table V). The presence of the two dihydric phenols,

TABLE VI

GROWTH OF THE FUNGI ON ARTIFICIAL MEDIA CONTAINING DIFFERENT AMOUNTS OF TANNIN IN ADDITION TO NUTRIENTS OF THE CONTROL MEDIUM. LINEAR GROWTH REPRESENTS DISTANCE FROM INOCULUM TO EXTREMITY OF MYCELIAL MAT; RATE OF THICKNESS REPRESENTS THICKNESS OF MYCELIUM IN TERMS OF AN ARBITRARY VALUE (PAGE 627). PERCENTAGES FOR EACH FUNGUS ARE IN TERMS OF THE GROWTH OF THAT SPECIES ON THE CONTROL MEDIUM*

TANNIN	*POLYPORUS BETULINUS*				*FOMES PINICOLA*				*POLYSTICTUS VERSICOLOR*			
MEDIUM	LINEAR GROWTH		THICKNESS		LINEAR GROWTH		THICKNESS		LINEAR GROWTH		THICKNESS	
%	*mm.*	%	*rate*	%	*mm.*	%	*rate*	%	*mm.*	%	*rate*	%
0.0*	20	100	2	100	25	100	3	100	42	100	5	100
0.01	23	115	2	100	25	100	3	100	44	105	5	100
0.05	22	110	2	100	22	88	3	100	38	90	4	80
0.10	18	90	2	100	20	80	3	100	18	43	2	40
0.15	15	75	2	100	19	76	3	100	0	0	0	0
0.20	15	75	2	100	20	80	3	100	0	0	0	0
0.30	12	60	2	100	12	48	4	133	0	0	0	0
0.40	7	35	3	150	12	48	4	133	0	0	0	0

* Control medium.

resorcinol and quinol, retarded the growth of *P. versicolor,* while *F. pinicola* and *P. betulinus* were retarded appreciably only in the presence of the methylated catechol, guaiacol.

Tannin at a concentration of 0.15 per cent. completely inhibited the growth of *P. versicolor,* while the other fungi continued to grow at a concentration of 0.4 per cent. (table VI). The substrate of *P. versicolor* turned to a deep brown color in the presence of tannin, a phenomenon which suggests that oxidation products of tannic acid may be a factor inhibiting growth. With the increase in concentration of tannin, the ratio of surface area over thickness of mycelium of *F. pinicola* and *P. betulinus* increased.

Growth on aqueous extracts of woods

Cultures were made on a series of media containing aqueous extracts of different woods. The control medium was the same as that used in preceding experiments.

Black and white birch extracts did not inhibit the growth of any of the fungi, in fact the development of mycelium was increased (table VII, fig. 1). On the other hand, white pine and osage orange extracts, both containing a

TABLE VII

GROWTH OF THE FUNGI ON ARTIFICIAL MEDIA CONTAINING AQUEOUS EXTRACTS OF DIFFERENT WOODS IN ADDITION TO THE NUTRIENTS OF THE CONTROL MEDIUM. LINEAR GROWTH REPRESENTS DISTANCE FROM INOCULUM TO EXTREMITY OF MYCELIAL MAT; RATE OF THICKNESS REPRESENTS THICKNESS OF MYCELIUM IN TERMS OF AN ARBITRARY VALUE (PAGE 627). PERCENTAGES FOR EACH FUNGUS ARE IN TERMS OF THE GROWTH OF THAT SPECIES ON THE CONTROL MEDIUM*

MEDIUM	*POLYPORUS BETULINUS*				*FOMES PINICOLA*				*POLYSTICTUS VERSICOLOR*			
WOOD EXTRACT	LINEAR GROWTH		THICKNESS		LINEAR GROWTH		THICKNESS		LINEAR GROWTH		THICKNESS	
	mm.	*%*	*rate*	*%*	*mm.*	*%*	*rate*	*%*	*mm.*	*%*	*rate*	*%*
None*	**20**	**100**	**3.0**	**100**	**25**	**100**	**2.7**	**100**	**41**	**100**	**4.0**	**100**
White pine	0	0	0	0	3	12	5.0	185	30	73	4.3	107
Basswood	15	75	3.3	110	20	80	4.0	144	42	102	4.7	117
White oak	12	60	3.0	100	8	32	4.3	159	36	88	5.0	125
White birch	20	100	3.7	123	23	92	4.0	144	45	109	4.3	107
Butternut	19	95	3.7	123	23	92	4.0	144	26	63	5.0	125
Osage orange	0	0	0	0	0	0	0	0	1	3	2.0	50
Sycamore	20	100	3.7	123	16	64	4.0	144	43	106	4.3	117
Black birch	25	125	3.7	123	27	108	4.0	144	43	106	4.7	117
Red cedar	18	90	3.7	123	25	100	4.0	144	38	92	5.0	125

* Control medium.

colloidal suspension unlike that in the other extracts, were strikingly inhibitive to the growth of the three fungi, especially to *P. betulinus,* and also but to a less extent, to *F. pinicola.*

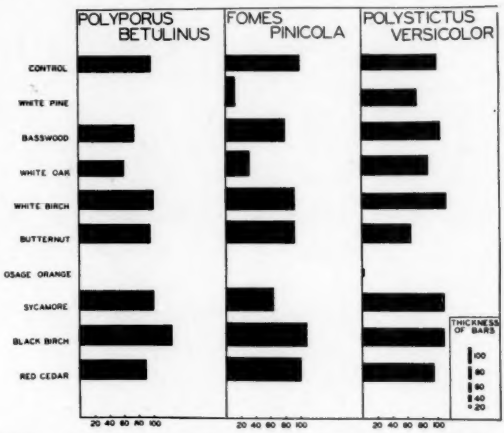

FIG. 1. Growth of the fungi on artificial media containing aqueous extracts of different woods in addition to the nutrients of the control medium. Length of bars represents linear growth, thickness of bars represents thickness of mycelium. Percentages for each fungus are in terms of the growth of that species on the control medium.

The reddish brown extracts of white oak, butternut, basswood, and red cedar differed in their influence on the development of the mycelium of each fungus. *P. versicolor* was retarded by extracts of white oak and butternut, yet it grew better than, or equally as well as, the other fungi on white oak, basswood, birch, and sycamore. Extracts of basswood, red cedar, and white oak definitely retarded growth of *P. betulinus,* while black birch extract supported better growth of the birch fungus than any other extract. *F. pinicola* was inhibited by basswood, white oak, butternut, and sycamore extracts. The best growth of this fungus occurred on black birch extract, although the organism grew better on red cedar than either of the other fungi, and better on white pine than *P. betulinus.*

GROWTH OF THE FUNGI ON WOODS

Polystictus versicolor and *Polyporus betulinus* were cultured on a variety of woods under laboratory conditions. Both fungi grew luxuriantly on sycamore, black and white birch, and basswood, but very poorly on white oak, butternut, white pine, osage orange, and red cedar (fig. 2). The first group of woods named were light colored while the latter group with the exception of white pine possessed dark-colored heartwood due to the presence

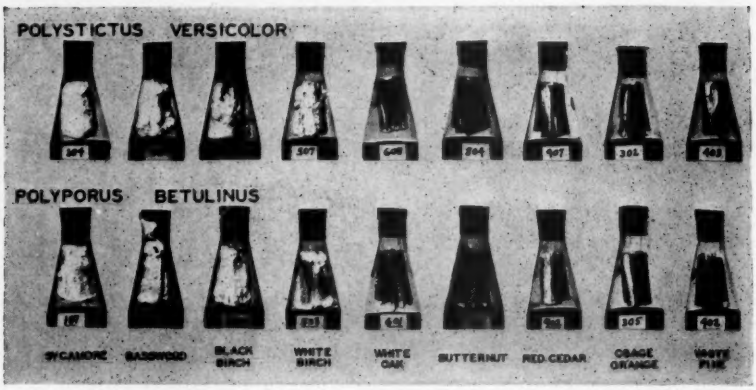

FIG. 2. Eight months old cultures of *Polystictus versicolor* and *Polyporus betulinus* on different woods.

of colored materials, and light-colored sapwood. The mycelia of the fungi were seldom found on the dark colored heartwoods, and then only very scantily. The light colored sapwoods invariably supported fair growth of mycelium, thus indicating that growth was retarded by the presence of substances found in the dark colored heartwoods of certain trees. The fungi did not differ markedly from each other in extent of growth, although the birch fungus produced less mycelium than *P. versicolor*.

Discussion

The chief problem in these experiments was the determination of outstanding nutritional differences and growth contrasts of *Polystictus versicolor*, *Fomes pinicola*, and *Polyporus betulinus* on artificial media, in order to gain a clue to the nature of the specificity of the latter two organisms for certain substrates. It was found that variations in the kind of carbohydrate, nitrogen, and mineral constituents of artificial media caused differences in the growth of the fungi, varying from mycelial mats spreading over the entire surface of the substrate to none at all, and from very thin to thick and luxuriant growths. The addition of phenolic compounds and aqueous extracts of woods to artificial nutrient media inhibited or increased in varying degrees the growth of each fungus. The activity of oxidases and reductases of the fungi in the presence of indicators was also different for each species. Growth of *P. versicolor* and *P. betulinus* was observed on a variety of woods but no specific differences between the fungi were noticed.

Difficulty in interpretating data and comparing them with those of other investigators was experienced because of certain variable factors which were believed to influence growth of fungi. Woods collected in different seasons,

or from different regions, and those dried or prepared for cultures under different conditions may cause significant variations in development of the fungi growing on them or on their extracts. Chemicals obtained from different sources and used in preparing nutrient media may influence differently the development of fungi. SCHOPFER (**14**) discovered that impurities present in some maltoses and absent in others were essential for reproduction of certain phycomycetes. Differences in growth characteristics observed by SCHMITZ (**13**) with strains of *Fomes pinicola* isolated from different wood hosts suggested the necessity of knowing the wood host from which the fungus was isolated and also the nature of the stock culture medium on which the fungus was grown previous to the experiment. Significant changes occurring in woods during the preparation of laboratory cultures and probably involving among other things leaching and chemical or physical modifications of wood substances not only may vary with different procedures used by investigators and thereby modify growth data, but also may cause growth phenomena in laboratory cultures which are different from those occurring in nature. Characteristics common for a fungus growing on artificial substrates, such as the agar gel, were regarded as not necessarily being in correlation with those of the same fungus growing on woody substrates which are more complex physically and chemically. Also considered very significant and probably affecting the development of the fungi were the differences in the environments of nature and of the sterile, humid, enclosed, and temperature-controlled laboratory culture chambers.

Polystictus versicolor was very profuse in its growth, and showed evidences of possessing much vitality and hardiness on a wide variety of nutrient media, and in the presence of many toxic substances, including glucosides, phenolic compounds, and extracts of woods. Neither *F. pinicola* nor *P. betulinus* showed such great adaptability to this variety of substrates. The coefficient of variability of growth over the surface of the control medium in these experiments was 3.7 per cent. for *P. versicolor*, while those for *F. pinicola* and *P. betulinus* were three to four times greater, 10.7 and 13.4 per cent., respectively (table VIII). The average linear growth of *F. pinicola* and *P. betulinus* was only 64 and 45 per cent., respectively, of the growth of *P. versicolor* on similar lots of control media. It becomes apparent, then, that *P. versicolor* can adapt itself to many different substrates while the other two fungi are much less adaptive and may be inhibited in growth by certain substances or combinations of substances present in the substrates.

LUTZ (**10**) reported that neither *Ungulina betulina* (=*Polyporus betulinus*) nor *Polyporus pinicola* (=*Fomes pinicola*) would grow on oak wood unless the wood were first extracted with water to remove substances inhibitive to the growth of the fungi. SNELL (**17**) and LIESE (**9**) found that *Polyporus betulinus* grew equally well and caused similar amounts of decay in

TABLE VIII

COMPARISON OF THE GROWTH OF THE FUNGI CULTURED AT DIFFERENT TIMES ON THE CONTROL MEDIUM. LINEAR GROWTH REPRESENTS DISTANCE FROM INOCULUM TO EXTREMITY OF MYCELIAL MAT; RATE OF THICKNESS REPRESENTS THICKNESS OF MYCELIUM IN TERMS OF AN ARBITRARY VALUE (PAGE 627)

CONTROL MEDIUM	*POLYPORUS BETULINUS*		*FOMES PINICOLA*		*POLYSTICTUS VERSICOLOR*	
	LINEAR GROWTH	THICKNESS	LINEAR GROWTH	THICKNESS	LINEAR GROWTH	THICKNESS
no.	*mm.*	*rate*	*mm.*	*rate*	*mm.*	*rate*
208	20	2	29	5	43	5
	15	2	28	5	44	5
	16	2	30	5	42	5
318	17	3	22	4	40	4
	20	3	23	4	42	4
	20	3	24	5	40	4
403	25	3	30	5	45	5
	24	3	30	5	45	5
	22	3	30	5	.....	...
620	20	3	24	2.7	42	4
	18	3	25	2.7	40	4
500	17	3	30	4	44	5
	17	3	30	4	43	5
	18	3	30	4	42	5
800	20	2	25	3	42	5
	20	2	25	3	42	5
Total	309		435		636	
Mean	19.3		27.2		42.4	
Percentage mean in terms of mean of *P. versicolor*	45		64		100	
Standard deviation	2.6		2.9		1.6	
Coefficient of variability	13.4		10.7		3.7	

laboratory cultures on oak, basswood, certain conifers, and birch woods. Similar results were obtained by the author with the same fungus species on sycamore, basswood, and birch which were in contrast with poor growth of the fungus on white oak, butternut, osage orange, red cedar, and white pine. That the substances present in woods and inhibitive to growth of certain fungi are water soluble and can be removed by water treatments was further suggested in the work of SOWDER (**18**), ANDERSON (**1**), and HAWLEY, FLECK, and RICHARDS (**6**) and was evident in these experiments from the exceptionally good growth of *P. betulinus* on aqueous extracts of birch and the definite

inhibition of growth by extracts of white oak, osage orange, red cedar, white pine, and basswood.

Water soluble substances in woods which are best known for their toxic effects on wood-destroying fungi are the phenolic compounds. Either *P. betulinus* or *F. pinicola* was inhibited to some extent by 0.02 per cent. α-naphthol, pyrogallol, resorcinol, and guaiacol in artificial media. Growth of these fungi was strongly retarded by 0.3 and 0.4 per cent. tannin. It follows that higher concentrations of tannin such as those present in some woods would also inhibit the growth of these wood-destroying fungi and thus be a factor responsible in part for the specificity of fungi for certain substrates (**2, 10**). *P. versicolor,* however, which is a common saprobe on many woods containing tannins, and thus apparently tolerant of phenolic compounds, was completely inhibited on artificial media in the presence of 0.15 per cent. or more of commercial tannin. An examination of the cultures of this fungus grown on substrates containing phenolic compounds or extracts of woods known to contain soluble phenolic substances showed an inhibition of growth of the fungus and a coloration beneath the mycelium of the organism, caused by the presence of oxidation products of the phenolic compounds. Since tannin and similar compounds present in substrates are inhibitive to the growth of the widely occurring organism, *P. versicolor,* as well as to that of the specific fungi, *P. betulinus* and *F. pinicola,* it follows that the presence of phenolic compounds is not the only factor which may determine the specificity of these fungi for certain woods in nature.

Glucosides, such as saponin and salicin, were not only in general inhibitive to the growth of fungi but they also varied in their toxic effects on each species. Growth of *P. betulinus* was completely inhibited by saponin, in contrast with the behavior of *P. versicolor.* Salicin favored relatively good growth of *P. betulinus,* and *F. pinicola* was retarded in growth by all of the glucosides. These observations not only suggest the inclusion of glucosides among the list of causal factors for specificity of fungi but also their very great importance as factors of growth and development of wood-destroying fungi on woods which may vary greatly in their glucosidic contents.

Differences in growth induced by various nutrients in artificial media were noticeable in cultures of all three fungi. *F. pinicola* exhibited relatively poor growth on carbohydrate media, especially in the presence of pentoses, galactose, and certain other carbohydrates which supported good growth of the other two fungi. The least variation in growth of this fungus on any series of nutrients was on media varying in kinds of nitrogen, where it grew equally well on inorganic nitrogen salts, amino acids, and proteins. In a contrasting manner, *P. betulinus* showed very good growth on the variety of carbohydrate media, but grew in varying amounts on the different nitrogen and mineral salt nutrients. Production of mycelium by

this fungus was especially less in the absence of potassium, phosphates, and nitrogenous compounds containing tryptophane, and in the presence of inorganic nitrogen salts and glycine, cystine, and tyrosin which were toxic in their effects on growth. These specificities toward nutrients which either inhibit or appear essential for good growth of the fungi in laboratory cultures suggest that a similar specificity may also occur on the widely different woody substrates in nature.

Studies of chemical changes in woods due to decay by fungi have been confined largely to soluble extractives, lignin, cellulose, and pentosans. The "proximate analysis" of apple wood rotted by *Polystictus versicolor* indicated that lignin was not attacked (**16**). However, BAYLISS (**3**), using microchemical methods, and CAMPBELL (**4**), using more precise analytical methods, distinguished *P. versicolor* from brown-rot fungi by its delignifying action on woods. CAMPBELL reported pentoses were removed first, then lignin was attacked, and finally, in the presence of increased acidity, cellulose. The rots produced by *Fomes pinicola* (**11**) and *Polyporus betulinus* (**7**) were described as brown rots in which relatively more cellulose than lignin was removed.

The majority of brown-rot fungi listed in a recent pathology text (**8**) were reported as occurring on gymnosperms, birches, and alders, and more than two-thirds of the white-rot fungi were described as commonly attacking angiosperms. Analyses, although incomplete for thorough comparisons of nutritive characteristics, show that gymnosperm woods differ markedly in chemical composition from angiosperm woods. To exemplify, an analysis of the data of RITTER and FLECK (**12**) and of SCHORGER (**15**) showed that the angiosperm woods analyzed by them contained among other and even greater differences 100 per cent. more pentosan than gymnosperm woods. Growth of the conifer fungus, *F. pinicola,* on pentoses and pentosans in artificial media was correspondingly much less than that of the two angiosperm-inhabiting fungi on similar media. These observations suggest that both quantitative and qualitative differences in nutrients existing among species of woods may be of considerable physiological importance to the successful growth and reproduction of fungi on woody substrates.

Since only three species of fungi were studied in these experiments, one cannot generalize too freely from the data. It is interesting to note, however, the correlation between the oxidation-reduction reactions of the fungi and their growth characteristics. *P. versicolor,* generally recognized as readily attacking phenolic compounds such as tannin and lignin, and being very wide spread in distribution on angiosperm and gymnosperm woods, possessed strong oxidases. *P. betulinus,* however, not extensively attacking tannin or lignin, and exhibiting a specificity only for birch woods, possessed strong reductases and no oxidases; while *F. pinicola,* which is specific for

gymnosperm woods but occasionally is found on certain angiosperms, possessed strong reductases and some oxidases. These data are indicative of the importance of respiratory enzymes in the phenomena of specificity of fungi for certain substrates which may lack essential respirable nutrients or contain toxic substances inhibitive to respiration and growth.

The differences in nutrition and growth noted herein are considered quite suggestive as to the causes of specificity among wood-rotting fungi. Needless to say, this cause is most likely a factor-complex involving physical, chemical, and biological characteristics of the substrates and the fungi. It was shown that the nature of carbon and nitrogen sources influenced the growth of wood-rotting fungi. The absence of certain nutrients or growth factors in woods may retard initial development and subsequent assimilative growth either entirely or to the extent that the fungus may never form a fructification by which the presence of the organism in woods is commonly recognized. Antagonistic substances present in woods may similarly affect nutrition, respiration, or reproduction. Principal among these antagonistic substances inhibitive to growth and studied in this investigation were glucosides and phenolic compounds.

Summary

1. The nutrition of *Polystictus versicolor* Fr., *Fomes pinicola* (Fr.) Cooke, and *Polyporus betulinus* Fr. was studied by observing the growth of these fungi on artificial media varying qualitatively in nutrients, and on nutrient media containing phenolic compounds, aqueous extracts of woods, and oxidation-reduction and pH indicators, and on woods.

2. Certain nutritional characteristics were common to all three species:

(a) Polysaccharides supported the best growth of mycelium. Pentoses generally were not utilized as efficiently as hexoses. No growth occurred on lactose.

(b) Amino and ammonia nitrogen was better utilized than amide, nitrate, and nitrite nitrogen. The best growth was observed on proteins containing relatively large amounts of glutamic acid and tryptophane. Amino acids containing phenyl and disulphide radicals retarded growth.

(c) Potassium and phosphate salts were essential for optimum growth. Phosphate was associated with increased thickness of mycelial mats, and potassium with increased linear growth of mycelium.

(d) Glucosides and phenolic compounds retarded growth.

(e) Aqueous extracts of white pine and osage orange were especially toxic, while those of black and white birch were non-toxic and even appeared to accelerate growth. Other wood extracts influenced the growth of the fungi in varying ways.

3. *P. versicolor* invariably grew luxuriant mycelial mats on artificial media, and showed only a little selection for kinds of nutrients. It utilized

both salicin and saponin as a source of carbohydrate, and was most tolerant to the greatest number of aqueous extracts of woods. Growth of *P. versicolor,* like that of *P. betulinus,* was relatively poor on inorganic and amino nitrogen media. Unlike the other two fungi, its growth was retarded by the presence of quinol and tannin.

4. *F. pinicola,* in comparison with *P. versicolor,* formed less abundant mycelial mats with a much greater variability on different media. It was especially sensitive to differences in the carbohydrates, growing poorest on media containing pentoses, pentosans, galactose, and sucrose, while carbohydrates containing glucose produced in general the best growth. *F. pinicola* grew relatively well on inorganic nitrogen salts, amino acids, and proteins alike. It was inhibited by both saponin and salicin, and, like *P. betulinus,* by guaiacol. Growth on aqueous extracts of woods was most variable, being best on red cedar and birch extracts.

5. *P. betulinus* produced relatively thin and often scanty mycelial mats and of all three species showed the greatest variability in growth. It was most sensitive to differences in nitrogen sources, only gliadin, Witte's peptone, casein, and glutenin producing a good growth of mycelium. Growth on carbohydrates was like that of *P. versicolor,* very uniform. Poor growth occurred in the absence of the less acidic potassium phosphates, and in the presence of guaiacol and saponin. Aqueous extracts of white pine and osage orange inhibited growth, those of white oak, red cedar, and basswood greatly retarded growth, and birch extracts accelerated growth.

6. *P. betulinus* grew equally well on heartwoods of sycamore, basswood, and birch, on sapwoods of white oak, butternut, and osage orange, and on white portions of red cedar wood. *P. versicolor* differed only in producing more luxuriant mycelial masses than the birch fungus.

7. The presence of a strong oxidase system was recognized in *P. versicolor* by its oxidation of tannin, resorcinol, quinol, tyrosin, and guaiacol. Oxidases were very weak in *F. pinicola* and not detectable in *P. betulinus.*

8. Reductases were especially abundant in *F. pinicola* and *P. betulinus,* but apparently weak in *P. versicolor.*

9. Possible causes of specificity of these fungi on wood in nature are the absence of essential nutrients, and the presence of toxic substances in woods which may influence initial development, respiration, or fructification of the fungi.

10. It was possible to distinguish physiologically between these (a) gymnosperm-inhabiting and angiosperm-inhabiting fungi, (b) white-rot producing and brown-rot producing fungi, and (c) fungi specific and those not specific for certain woods in nature.

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METHODS OF SAMPLING VISIBLE RADIATION[1]

RAYMOND H. WALLACE
(WITH TEN FIGURES)

Introduction

There is perhaps no factor in the measurement of light which is more essential to the correct evaluation of light records and their correlation with various biological phenomena than the method of sampling of this radiation. Yet a survey of the literature on the subject discloses that no comprehensive tests have been made on the various possible physical arrangements of the sensitive surfaces of the receiving units and the degree of similarity which these various combinations will exhibit when checked by simultaneous readings of the same light station. We are all aware that the method so commonly used of exposing a single-plane receiving surface in one direction gives erratic results. Thus any one that has used any of the numerous exposure meters or illuminometers, having a flat surface as the receiving unit, knows from experience that a reading may vary from a few to perhaps almost 100 per cent. if the receiving unit is tilted a few degrees. This indicates a fundamental flaw in the sampling, for the original value will be found when the first position is again obtained. It would seem, therefore, that we must, if we are to use light records with any degree of success, have methods of sampling that make impossible these great fluctuations in value.

AURENS (1) working on the light habitats for various regions in Sweden, has taken great care to insure that the sampling method used checks perfectly with the method in use by the weather bureau for their determinations of total radiation. In his test the photocell is mounted in a chamber having a single round window in the top to admit the light to be measured. This window is fitted with a piece of opal glass which is ground on both sides to insure that the optics of the arrangement obey Lambert's cosine law. This unit is, of course, mounted horizontally in accordance with weather bureau practice. His method can be said, therefore, to duplicate the weather bureau practice with an instrument intended to measure visible radiation for use in work on silviculture and ecology. Apparently no tests were made to determine the suitability or dependability of this method for work of this type. It is obvious that a method which may be perfectly adapted to determinations of solar constants and other highly specialized factors of interest to those in meteorology may be quite unsuited for ecological or physiological studies.

[1] This paper was presented before the symposium on the measurement of light, held under the auspices of the New England Section of the American Society of Plant Physiologists at Connecticut State College, May 8 and 9, 1936.

The possible lack of suitability of radiation records of this type as applied to silvical research has been recognized by GAST (2) in his studies on the effect of total radiation on the growth of pine. He states as follows: "Further, in order to give a time record it (the receiving unit) must integrate the rays coming from all directions at normal incidence." In attempts to attain this condition GAST has designed and constructed four types of radiometers made up of different combinations of spherical junction thermocouples. His best type, type D, integrates the radiation with an accuracy of $\pm$ 9 per cent. for angles of incidence from 5° to 80°. For the entire range from 0° to 90° the values are as follows: For 90° the relative sensisitivity is 74; for 60°, 78; for 30°, 86; for 10°, 94; for 5°, 95; and for 0°, 100.

It is in response to this need of more definite data than are now available on methods of sampling that the series of tests reported in this paper were made. I shall limit myself in the present paper to sampling of radiation from a light station which is unobstructed by surrounding objects and therefore has the horizon for the limit. The data will, however, be for all types of weather from days on which no clouds were in the sky to days that were completely overcast.

Materials and methods

Discontinuous hand readings are of little value in studying a problem of this sort. It was necessary therefore to obtain continuous simultaneous records from the different types of sampling units, and to check one against the other for accuracy.

An entirely new recording potentiometer of the duplex type was constructed for the work. The instrument was similar in many respects to the one exhibited at Pittsburgh in 1934 and identical to the one exhibited at St. Louis in 1935, except that the new instrument was a double one with two completely separate though identical recorders built into one cabinet and writing their respective records on one drum. Full details and instructions for the building and operation of this instrument can be found in the preceding issue of this journal (WALLACE, 3). Suffice it to say here, however, that this instrument is ideally adapted to the recording of light, due to several specific characteristics.

In the first place the bridge adjustment is practically universal, in that any millivoltage from 30 to 1500 can be obtained by the turn of a dial. This enables one to set up any given light intensity so that it will occupy the entire recording drum or any portion thereof. Also there is a universal control of the zero point of the bridge, so that one can place it at any point between the two ends of the drum. Therefore, when one uses a duplex recorder of this type, he can set up any two photounits for equal centimeter deflection irrespective of the actual millivoltage outputs of the units themselves. Also because of the zero adjustment he can set the two units so that their base lines

coincide on the drum, or place them 1 cm. apart or in any other combination that may be desirable. All curves given in this paper were obtained on this instrument.

Most of the data given below were obtained during the period from April 16 to May 6, 1936. The light station for all of the tests was two adjacent ventilators 4 feet high located about 15 feet apart on the roof of the laboratory. This position completely avoided shading of the photounits at any time. No adjustment whatever, except in figure 5, was made during the progress of a test. All tests on each combination of photounits were run for at least three days in succession, or until the type of curve could be predicted. All figures given in this paper are unretouched photographs of the records themselves. No actual foot-candle values for the curves are given, since it was felt that one is chiefly interested in the exact comparative values shown by the two units and that it was best not to complicate it.

The photocells used throughout these tests were the Weston photronic. The photocells were never exposed to the direct rays of the sun except on the occasions mentioned in the paper. As will be seen below, it is very necessary to take this precaution to avoid damage to the cells. The spheres mentioned in the paper were the common type of opalescent shades obtainable at any good electrical store and had transmission factors of 48 and 52 per cent. respectively. Two sizes were used, the small 8-inch size for the one-celled photounits, and the 12-inch size for the multiple-celled photounits.[2]

All photocell units were attached to rubber-covered wires which led down over the roof to the laboratory below where the recorder was in operation. Each photounit was likewise drained with a 10-ohm resistance placed directly across the lead wires at the imput terminals of the recorder. This was to insure linearity between the current output of the cells and the light intensity.

The usual method of setting up the instrument for the different combinations was as follows. If possible each new series involving a new photounit combination was begun about noontime on a clear day. This enabled one to set the two units for identical centimeter deflection on the recorder drum. Setting up units for the first time in the evening or morning is simply guesswork and almost invariably results in lost time. If, however, they are set for equal deflection at normal or almost normal incidence at noontime, not uncommonly the records for the first day are just as perfect as any subsequent ones.

[2] The spheres can be the size best suited to your convenience since the sampling of radiation is independent of the size of the sphere used. Thus a series of determinations of a certain light intensity with one photocell under spheres of 5, 6, 7, 8, 10, and 12 inches respectively gave identical values when one corrected for the difference in transmission of the different spheres. The globes must be the white opalescent type and not those made of ground glass. One of the latter type was tested and found to give an error of 500 per cent. in the same test (series 7 below) in which the opal sphere gave a 5 per cent. error.

The setting of the zeros was adapted to the test series being run. It was sometimes not possible to forecast just which zero combination would best show the agreement or lack of agreement of the different photounit combinations. It was necessary therefore in these cases to set up the more desirable zero combination after one day's record was obtained. The zero combination found to be best suited to most of the photounit combinations was that in which one zero was at the very base of the drum and the other 1 cm. above it. It must also be remembered that the time axis for the two curves is not the same. The upper curve is always 5 minutes behind the lower curve. One must bear this in mind, since the exact centimeter deflection of any point on a record is the vertical distance of that point above the zero line for that curve. For most purposes, however, the distance between the same points of the two similar curves will give the agreement or lack of agreement between the two curves with sufficient accuracy.

Experimentation

Types of photocell combinations

The more significant physical arrangements or orientations of the sensitive surfaces of the photocell were tested in these experiments. The ideal shape of the sensitive surface, if perfect sampling of radiation is to be obtained, would seem to be that of a sphere. This unit would sample the radiation falling on a point, and consequently be independent of angle. However, at the present time there are no photocells made with spherical receiving surfaces and possibly there may never be because of physical difficulties involved, and consequently this ideal cannot be reached.

The next most perfect sampling device would seem to be a unit in which a great number of sensitive surfaces of equal value and size would face in all possible directions. This condition is likewise very difficult or impossible to attain.

Type A.—A compromise was made with these conditions in the following experiments by placing a photocell on each of the six sides of a wooden cube and supporting this on short wire legs so that the center of the wooden cube was about 6 inches above a base support. A spherical shade, 1 foot in diameter, was then placed over the unit so that the center of this globe and the center of the photounit built around the wooden cube would coincide. We have in this combination a six-faced-sampling unit in the center of a large integrating sphere. Physically this unit should very adequately integrate the light falling on a point, since its sensitive surfaces face in all directions and the integrating sphere will tend to equalize the light and reduce errors due to unequal sensitivity of the individual photocells. The same unit without the integrating sphere was tested later and found to be quite inferior to the unit with the sphere, as described below. This six-celled unit under the inte-

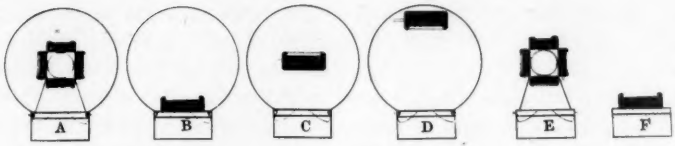

FIG. 1. Schematic diagram showing the arrangement of the different types of photounits: A. Six photocells fastened to the six sides of a cube and thus facing the four cardinal points, zenith, and downward, and enclosed under an integrating sphere; B. One photocell facing up from the base of an integrating sphere; C. One photocell facing up from the middle of an integrating sphere; D. One photocell facing downward from the top of an integrating sphere toward a reflecting surface in its base; E. A six-celled photo unit similar to A, except used with no sphere; F. A one-celled unit similar to B, except used without a sphere.

grating sphere is the one against which all other combinations were checked (fig. 1, A).

TYPE B.—A second type of photounit consisted of a single photocell facing up from the base of an opal diffusing-sphere. The sampling characteristics of this unit will be determined almost completely by the integrating characteristics of the globe itself, that is, the accuracy of the physical summation of the radiation falling on a point (fig. 1, B).

TYPE C.—A third type was a single photocell facing up from the center of an opal diffusing sphere. The sampling characteristics of this combination will depend to some extent on the photocell being up in the center of the sphere instead of at the base of the sphere, but to a greater extent in the integrating properties of the sphere itself (fig. 1, C).

TYPE D.—A fourth type consisted of one photocell fastened in the top of a sphere and facing downward toward a white, reflecting surface in the base of the sphere (fig. 1, D).

TYPE E.—A fifth type was the six-celled unit mentioned as type A but used without an integrating sphere, so that the cells were exposed to the direct rays of the sun (fig. 1, E).

TYPE F.—A sixth type consisted of one photocell mounted horizontally and facing the zenith and not covered by a diffusing sphere. This is the method commonly used by the Weather Bureau for sampling total radiation by means of pyrheliometers (fig. 1, F).

EXPERIMENTS ON PHOTOUNIT TYPES

A series of tests was run for each of the types of photounits given above, and on certain other factors that affect the accuracy of sampling. As mentioned above, the light stations used in the tests can be considered as identical. I shall now survey the results of these tests.

SERIES 1. CAN IDENTICAL RECORDS BE OBTAINED.—The experiments of series 1 were designed to test all factors entering into the sampling of

light; the adequacy of the recorder, the freedom from stray electrical pick-ups, the use of identical photounits, the shape of the integrating shade, and any other factors which might prevent identical curves being obtained from what seemed to be identical sets of conditions. It was felt that identical records from two entirely independent but identical photounits, measuring the same light station at the same time on two entirely separate electrical circuits, connected to two entirely separate recording mechanisms writing on the same drum, would indicate that all factors entering into the tests were satisfactorily controlled.

This experiment was first run with type B photounits—that is, one photocell mounted in the base of each shade and facing zenith (fig. 1, B). The shades used were, however, not spheres as was originally planned since they were not available at the moment. Two other opalescent shades such as are shown in figure 2, A were substituted temporarily. The units were mounted

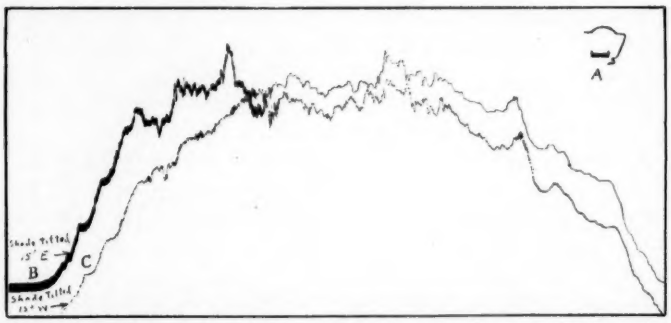

FIG. 2. Records for April 9, 1936, showing the effect of shape of integrating shade on the sampling of light. A. Shape of shade used; B. Curve for unit number one tilted 15 degrees to the east; C. Curve for unit number two tilted 15 degrees to the west. See text for further description.

on the ventilators and the bridges of the recorder set for equal centimeter deflections from zeros 1 cm. apart.

At the end of 3 days it was obvious that the two units were not sampling alike. Nevertheless, the discrepancy between the two records was consistent and duplicated each day so that it was possible to predict what the disagreement would be. After examining the duplicate records for a 3-day period it seemed obvious to me that the shade on unit number one was tilted to the east. Investigation revealed that not only was the shade on unit number one tilted 15° to the east but also that the shade on unit number two was tilted 15° to the west. The actual relationship of the two curves for the third day of the test is shown in figure 2. It will be noted that the two curves tend to diverge during the forenoon, run along fairly well together during the mid-

day period, and converge during the afternoon. Reference to figure 2, A, showing the straight-sided shades used as integrating units, will show that if unit one is tilted to the east and unit two tilted to the west the discrepancy found between the two curves in figure 2 would occur.

It is obvious from these results that it is not safe to use diffusing shades that are other than spherical in shape. The variation that showed up here, due to the two units not being level, proves there is unequal sampling of the light. Subsequent tests have shown, on the other hand, that little or no care need be exercised, even to the leveling of photounits, under spherical shades.

The test was repeated the following day with spherical shades. The curves obtained are shown in figure 3. It is almost impossible to find devia-

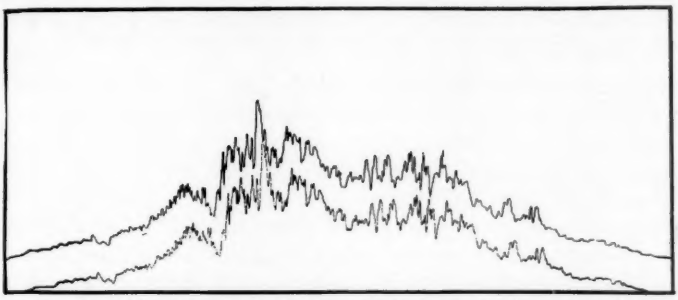

FIG. 3. Records for April 10, showing identical curves when spherical integrating shades were substituted for the type A of figure 2.

tions on the extreme points of more than 1 or 2 per cent., while the major portion of the curves is identical. It seems obvious from these data that the factor that had prevented identical curves in figure 2 is the type of shade used. One can answer therefore that identical records can be obtained from two identical photounits under the set of experimental conditions mentioned above. We are ready, therefore, to test the actual differences between the sampling from different types of photounits.

SERIES 2. SIX-CELLED UNIT *vs.* ONE-CELLED UNIT, TYPE B, BOTH UNDER DIFFUSING SPHERES.—This series of experiments was run to test type A photounit—the six-celled photounit with one cell facing each of the four cardinal points, zenith, and downward, against type B photounit—a single cell at the base of a sphere facing up. Figure 4 shows the curves from this combination of photounits. The upper curve is the six-celled unit, the lower one the one-celled unit. The zero for the six-celled unit curve is 1 cm. above that for the one-celled unit. It will be noted that during the later forenoon there is a period of about two hours with full sun (smooth part of the curve), followed by another two-hour period during which the light intensity is considerably

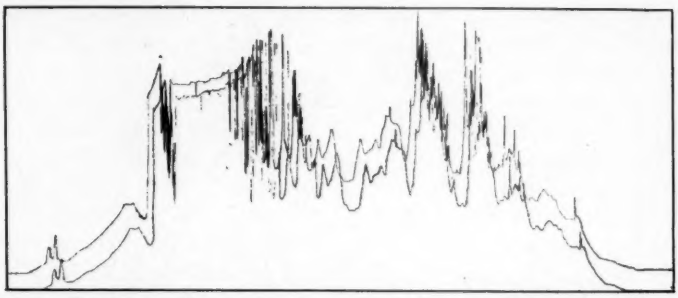

FIG. 4. Records for May 2, showing curves for type A and type B photo units in sampling of light. The upper curve, the type A or six-celled unit; the lower curve, type B or one-celled unit. Light conditions variable from heavy fog in the early forenoon to full sunshine just before noon, and variable light and cloud for the afternoon.

greater than that for the preceding period of full sun with no clouds. This increase of light intensity above that for full sun with no clouds is very common when there are large white clouds, and often appears on the records as close-spaced vertical lines such as are observed here. For the next 4 or 5 hours the sky was heavily overcast with no direct sun at all, followed by two periods when the sun again broke through for a short time to be followed by another period of completely overcast sky until dark. It is not necessary to interpret which curve is correct since the curves are almost identical. The maximum error of the extremes is about 5 per cent., while the major portion of the two curves is identical.

In figure 5 are shown the same two units on a very heavily overcast day. In this case the zero for the two curves is the same base line and the curves show up as parallel tracings with the curve for the six-celled unit 5 minutes back of that of the one-celled unit. The two curves do not agree well during

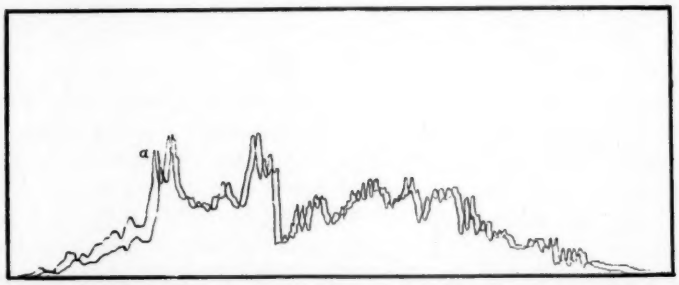

FIG. 5. Records for May 4, showing the sampling from the same combination as in figure 4, on a very heavily overcast, foggy day with no direct sun at all. The six-celled unit is the one lagging back of the other curve. The two units were set for equal amplitudes at point *a*; the lack of agreement prior to this point should therefore be ignored.

a part of the forenoon owing to the two units being set for unequal amplitudes. These were set equal at point *a*, and the agreement from here on is very close indeed.

The light conditions covered in figures 4 and 5 are for all extremes from full sunlight, through the marked fluctuations characteristic of alternate sun and shade with large white clouds, on to very heavily overcast sky with no sun at all. Throughout this great range of conditions the sampling of the two units is practically identical, the variations of the extremes being no more than 5 per cent. It would seem therefore that for all purposes, except those necessitating extreme accuracy, a type B photounit consisting of a single photocell facing up from the base of a sphere is just as adequate as the six-celled unit. This is very fortunate because the multiple-celled unit is very awkward to carry and difficult to use under field conditions where there is brush or other obstruction.

SERIES 3. SIX-CELLED UNIT *vs.* ONE-CELLED UNIT, TYPE C, BOTH UNDER SPHERES.—The difference between this series and the one just preceding is that in this case the one-celled unit is of type C, in which the single cell is placed up in the middle of the diffusing sphere facing upward, instead of in the base of the sphere facing up. The agreement between the two curves was good but somewhat inferior to that found in series 2 above. The maximum error of the extremes being about 15 per cent. as compared to the 5 per cent. in the other case. There is really no advantage whatever in this combination over the one cell in the base of the sphere facing up, so no further consideration was given it.

SERIES 4. SIX-CELLED UNIT *vs.* ONE-CELLED UNIT, TYPE D, BOTH UNDER DIFFUSING SPHERES.—In this series the one-celled unit was type D with the one-cell

FIG. 6. Records for April 22, showing the sampling characteristics of type A photo unit and type D unit, the one cell in the top of a sphere facing downward. Upper curve, the six-celled unit; the lower curve, the one-celled unit. The day was completely clear except for a few small clouds just before noon. The two rather conspicuous dips in the curves just prior to the period of small clouds were due to a high haze, scarcely visible to the eye, which passed over at that time. This haze was quite visible when viewed with amber glasses.

fastened in the top of the sphere facing a reflecting surface in the base of the sphere. This combination gives a fairly good agreement for the early forenoon and the late afternoon as can be seen in figure 6. Then there is a period during both the forenoon and the afternoon when the curve (the one with the lower base line) goes about 15 per cent. too high. This can be readily seen in figure 6 if one raises the lower curve up so its base line coincides with the base line for the six-celled unit. This period of excessive height gradually gives way to normal and then becomes lower and lower and reaches its point of maximum divergence from the six-celled-unit curve at noon. The curve, in other words, is badly "sway-backed" during the middle of the day.

This is just the type of curve that would be expected from type 4 unit as can be easily seen by examining figure 1, D. During the early forenoon and late afternoon when the sun is not high a greater amount of the light hitting the sphere will reach the photocell. Then during the middle of the day the maximum light will be hitting that portion of the diffusing sphere against which the back of the photocell is mounted. This blind spot will naturally cause the curve to be low for that period. All subsequent tests have shown this fundamental defect in the curves for this unit. This type D unit is not to be recommended.

SERIES 5. SIX-CELLED UNIT UNDER SPHERE *vs.* ONE-CELLED UNIT, TYPE F, WITHOUT DIFFUSING SPHERE.—This method of exposing the sensitive surface of the receiving unit horizontally to and unprotected from the direct rays of the sun is the standard method in use by the Weather Bureau for the determination of total radiation by means of pyrheliometers. It is theoretically possible for one to correct such records for normal incidence of the sun, that is, right angle or noonday incidence. One can do so, however, only when optical conditions are perfect, for only then does Lambert's cosine law apply. With the photocells this condition is only approximated, the disagreement being as much as 30 per cent. for low angles of incidence according to the technical data given by Weston for their cells.

Figure 7 shows the type of agreement between these two methods of sampling. It will be noted that the two curves are set on the same base line and have equal deflection at midday. During the forenoon the records show alternate sun and clouds and very poor agreement between the curves. There is a general tendency for the lower curve of the horizontally exposed cell to follow the cosine law but there are marked variations from it as will be noted by comparing points a and a_1 and other pairs of points in the same general region. Then the two curves approach each other rather closely at b. It is to be observed here, however, that the amplitude of the change of the six-celled curve at b is much less than it is for the uncovered cell. In the region of point c the situation is worse, since the curves now cross each other. At d the six-celled unit curve rises strongly while no such

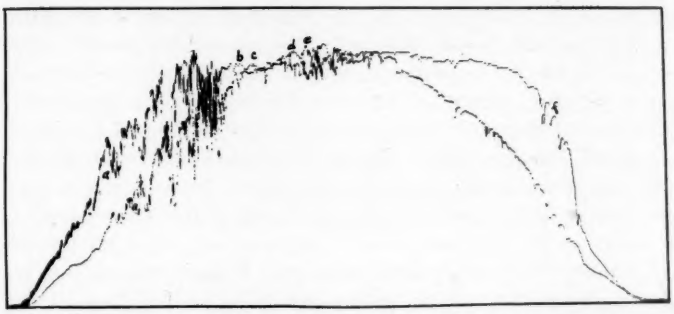

FIG. 7. Records for April 27, showing the sampling by type A unit and type F unit. The latter type is a photocell mounted horizontally and exposed directly to the rays of the sun. The upper curve, the six-celled unit; the lower curve, the one-celled unit. The forenoon was alternate light and cloud; the midday period slightly cloudy; and the afternoon almost completely clear. For further details see the text.

rise is evidenced by the curve for the other unit. The extreme situation is reached, however, at point *e* where the two curves are actually mirror images of each other. In the afternoon period, which was entirely clear except for a few scattered clouds, the true divergence of the two curves is plainly shown. It will be noted even here, however, in the region of *f* that light changes may be picked up by the six-celled unit which have little or no effect on the other unit.

Is it possible to correct the single-celled unit record to that for the six-celled unit with any degree of accuracy? Theoretically this can be done, as has been mentioned. That is, if there had been no clouds or haze at all during the afternoon and if the photocell obeyed the cosine law exactly, the correction would raise the lower curve up to match the upper curve. Since, however, there were clouds and also since the photocell obeys the cosine law only fairly well, such a correction is of little value. Moreover corrections for the horizontally mounted cell involve laborious calculations before the records can be used.

Much more important, however, than the failure of the photocell to obey the cosine law, or the work involved in the correction of the curves, is the fact that the six-celled unit and the horizontally mounted naked unit do not sample radiation alike even during midday when sun incidence is normal. This indicates a fundamental defect for which there is no correction. Refering again to points *b*, *c*, *d*, and *e*, it will be recalled that all possible combinations from difference in amplitude for a given light variation to a complete reversal or mirror image for the two curves is shown. This midday discrepancy is due to the presence of large white clouds. It has already been mentioned that very frequently large white clouds will increase the light

intensity far above that for a perfectly clear day. These clouds, depending upon their position in the sky, may affect the two sampling units differently. If they are in a low-lying bank they will record in full on the six-celled unit and yet may fail to register at all on the horizontally mounted unit. This has often been observed in such instances as that indicated by points *b*, *c*, *d*, and *e*. Again, one large cloud passing between the unit and the sun must affect the naked cell very strongly but may not greatly affect the other unit. In case the rest of the sky is free of clouds, the drop in the curves for the two records may be quite similar, but in case several other highly reflecting clouds are along the horizon then this record may show no drop at all since points of higher intensity counteract points of lower intensity. This physical situation can be well illustrated by the contrast in sharpness of shadows on a perfectly clear day with the same shadows on a day with white haze and highly reflecting clouds. The shadows in the latter case are much less dense due to the reduced relative importance of the direct rays of the sun. If then one is to apply Lambert's cosine law with any degree of accuracy to records of this sort, he must correct not only for the direct rays of the sun but also know the angle, duration, and intensity effects given by each cloud of importance in the sky and correct for them. This is, of course, impossible and it is just as impossible to unscramble records of this type.

Another point that must be brought out is that when the day is overcast enough it is immaterial whether integrating spheres are used or not. Thus

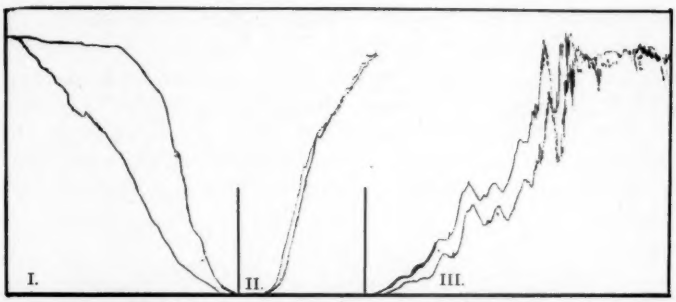

FIG. 8. Records giving a summary of the sampling by type A and type F units as shown on three successive days. I. Records for the afternoon of April 28, showing the divergence of the two curves as expected from the cosine law on a perfectly clear afternoon. II. Records for the forenoon of the day following, April 29, showing the agreement between type A sampling and type B. Type F was converted into type B merely by placing the sphere over it. III. Records for the forenoon of the following day, showing the sampling for type A and type D units. Part of this forenoon was heavily overcast, so the two units sample almost exactly alike (the two units are not set for identical amplitudes which causes the curves to drift apart, but this does not count. When, however, the direct sun breaks through along the flat part of the curve later, the sampling is similar to that shown at *b*, *c*, *d*, and *e* of figure 7.

in III of figure 8, we have the six-celled unit with its sphere and the horizontally mounted single cell with none. This pair of records has been plotted on the same base line but unfortunately were not set for equal amplitudes. If one allows for this difference in setting then the very close agreement between the two units becomes obvious. The uniformly overcast sky has now become the integrating unit and consequently the records agree. Notice how quickly, however, discrepancies appear when the sun breaks through at the top of the curves; the same situation as that along points *b*, *c*, *d*, and *e* of figure 7.

Another interesting point is presented in figure 8, I, which shows the typical curves for the naked photocell and the six-celled covered unit on a clear afternoon. Then figure 8, II, shows exactly the same conditions for the next forenoon, except that both units are now covered. The addition of the sphere brings the two units into perfect agreement on sampling, in the same manner as shown earlier in text figures 4 and 5.

SERIES 6. SIX-CELLED UNIT WITHOUT INTEGRATING SPHERE, TYPE E *vs.* ONE-CELLED UNIT WITH SPHERE.—In this combination, the situation was just reversed from that in the preceding test. The one-celled unit was now under an integrating sphere while the six-celled unit was not. In this combination the agreement is fair but the variations were as much as 15 per cent. This discrepancy is undoubtedly due, in great part, to the unequal sensitivity of the six photocells. That is, in case the strongest radiation is coming from the direction where the most sensitive cell intercepts it the curve will be high or vice versa in case a weak cell is in that position. Also the sampling of each passing cloud will be too high or too low depending on the photocell intercepting the light. The integrating sphere would have smoothed out most of this. In this combination also the curves become identical when the sky is heavily overcast. Another serious objection to this combination is the probable damage to the cells from exposure. This latter point is taken up in greater detail below.

SERIES 7. PHYSICAL CHARACTERISTICS OF PHOTOUNITS A AND B.—After the experiments reported above were complete, it seemed desirable to determine the physical characteristics of the two most dependable types of units. The six-celled unit (A) and the one-celled unit (B) were therefore tested in the darkroom. One of these tests was to place the unit on a turntable at a given distance from a 1000-watt Mazda bulb and make a reading. The table was then turned 90° and the next quarter tested and so on until all four were compared. The unit was then placed on a tilting device that enabled one to expose the unit to light from any angle without modifying the distance from the light.

In table I are given the data for the six-celled unit. The first set of tests

TABLE I

PHYSICAL CHARACTERISTICS OF TYPE A PHOTOUNIT*

CELL NO.	READING WHEN FACING LIGHT	
	WITHOUT SPHERE	WITH SPHERE
1	89.0	94.8
2	89.1	94.8
3	95.6	97.4
4	100.0	100.0
5	89.0	97.0

* The unit was revolved around its vertical axis to give the readings for cells 1, 2, 3, and 4 and turned on its side for cell 5 which was the cell that normally faced up. All cells are expressed in terms of the strongest one, number 4.

was made without the integrating shade. It will be observed that when the successive cells faced the light the readings differed, owing to the unequal sensitivity of the cells. When the shade was put on these, differences in sensitivity dropped to about one-half their former value owing to the integration of the globe. When the unit was tilted the reading obtained with cell 5 facing the light was about the average given for the four preceding cells. This is what would be expected from the combination. The type A photounit is shown by these data to have an error of about 5 per cent. when predominantly unilateral light is directed in succession toward the four photocells. This variation is only half as great as that for the four photocells exposed without the sphere. With matched cells this error should disappear.

When the unit with a single cell facing up from the base of a sphere was revolved about its vertical axis no variation of more than 0.1 per cent. was found. When the same unit was tilted through an angle of 180°, 10 feet in front of a 1000-watt Mazda bulb the maximum variation found was 5 per cent. On closer testing this variation was found to occur only when the angle of incidence was 18° or less. In other words, after the unit was tilted 18° toward the source of light the reading would remain constant for the rest of the 144° arc, or until within 18° of complete inversion of the unit. Even within the 18° angle where the variation occurred the error was merely progressive, first showing up at 18° and reaching 5 per cent. at 0°. This 18° portion would represent approximately 1 hour at sunrise and 1 hour at sunset when the record might show as much as a 5 per cent. error. For the rest of the day, no error of consequence would occur. This sampling would seem to be adequate for all except the most exacting problems.

SERIES 8. DEPENDABILITY OF PHOTOCELLS.—During the progress of these studies several points of importance to those working with photocells were

encountered. The first and perhaps the most important is the detrimental effect of direct sunlight on the Weston cell. This trouble first appeared in some further tests of type E photounit in which the cell is exposed directly to the full rays of the sun. This cell after several days of hot clear weather became completely erratic. Within a few minutes after it had even been set for equal deflection with the other unit it would be off-scale or have dropped to half of its former value. It was reset seven times in a period of 4 hours in an unsuccessful attempt to keep it on scale. It was then allowed to run for several days, set for only a small part of the full scale of the bridge. It immediately showed the following effects. During the early morning and late evening it usually gave a curve which agreed very well with the other unit but just as soon as the temperature reached a certain value the curve became erratic. The cell was then laid aside as being defective.

The next appearance of this trouble was during the last days that series 6, described above, was run. In this series the six-celled unit was tested without the diffusing sphere. At first good agreement in curves between this unit and the unit under the sphere was obtained, but suddenly the six-celled unit became very erratic. The results are shown in figure 9. The upper

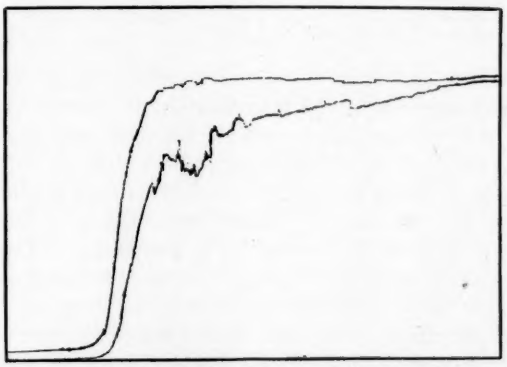

FIG. 9. Records for May 16, showing defective sampling by type A, the lower curve, as against type B the upper curve. These two types had always given agreement before. The type A unit had, however, been exposed for several days directly to the rays of the sun without the protecting sphere. It was possible from this curve to forecast that the photocell facing east had been badly damaged. See text for full description.

curve is for unit B and the lower for unit A. It will be observed that the two units start off in the early morning with very close agreement between the curves. But, as mentioned above, the curve suddenly becomes erratic when a certain light or temperature level is reached. The curve then slowly climbs and, as other cells of the unit receive the light, finally reaches the

value it should about noon. For the rest of the day (not shown in the figure) the curves agreed quite closely.

On the basis of this it was obvious that something was wrong with the photocell facing east and possibly the one facing south, but that the ones facing zenith and west were all right. The unit was removed from the roof and taken into the darkroom for recalibration of the individual photocells. This check-up, on May 28, was almost exactly one month later than the last calibration of the cells, on April 27. The results of this check-up are shown in table II. The cell facing east had dropped to 17.3 per cent. of its April

TABLE II

CALIBRATION OF PHOTOCELLS*

DATE	NUMBER OF CELL					
	1 (EAST)	2 (SOUTH)	3 (NORTH)	4 (WEST)	5 (ZENITH)	6 (DOWN)
April 27	100.0	85.7	100.0	80.0	80.0	80.0
May 28	17.3	41.7	80.0	80.0	80.0	80.0
May 28 Cells wedged	100.0	85.0	100.0	unchanged	unchanged	unchanged

* Sensitivity expressed in terms of cell 1 as 100.

27 value. The one facing south had dropped to 41.7 per cent. The one facing north was 80 per cent. and the remaining three were apparently normal. The method of checking these values was to expose the cells in succession to a 1000-watt bulb at a distance of about 18 inches, which gave about 1000 foot-candles. It was then assumed that those cells retaining their relative sensitivity were unchanged. Subsequent tests have borne out these assumptions. If the tests are run rapidly to compensate for voltage fluctuations and repeated several times, the results are accurate to within 5 per cent., which is adequate for comparisons of this sort.

When it was found that one cell had dropped to 17.3 per cent., in terms of the value of the three unchanged cells, it seemed reasonable that the trouble might be due to loose contacts within the cell case since the glass face itself was quite loose. To test this three small match-stem wedges were slipped between the glass face and the composition case. The cell immediately came back to full calibration value and remained there (table II). The same procedure on the other two cells that were defective brought them back to full value. Similar treatment of the three normal cells failed to change their sensitivities.

It was now obvious that something was defective in the contact system within the case. The poorest cell was then placed on its back in a crucible full of acetone for an hour to dissolve the cement and allow the back to un-

screw. These cells are made up of a metal disc on one side of which a metallic material is deposited. The back of the disc is one electrode and the front the other. On the front of the cell between the glass face and the face of the disc is the front collector or electrode-ring contact. The fingers of this ring are visible around the edge of the face. Another ring just like this one fits against the back of the photocell disc. The composition back is supposed to screw down and hold these two rings in contact with the disc. The rings are, however, very thin and have, at most, not more than 0.5 mm. to allow for contraction and expansion. Apparently when the cell is exposed directly to the rays of the sun the case is stretched by the pressure developed by the heat and on cooling part of the stretch remains. As a result the cells may become erratic on exposure to the sun.

It seemed reasonable that if suitable constant-pressure springs were utilized to maintain the electrode contacts, these defective cells might be fixed. To accomplish this a small hole was drilled in the exact center of the screw-back of the cell. A clock spring was cut into short lengths, two of which were fitted at right angles across each other with a hole drilled in the middle of them.[3] These were mounted on the screw-back with a small bolt. A thin bakelite disc, the diameter of the photocell disc, was placed against the back electrode ring for the springs mechanism to push against. This disc was necessary to avoid damage to the photocell. Part of the flange of the screw-back which was formerly supposed to hold the electrodes in place was cut off to give room for the spring legs to work. The unit was assembled by screwing the back in as usual. The first cell (the one that had dropped to 17 per cent. normal) worked perfectly so the two other defective ones were fixed. The three then had the same sensitivities that they had on April 27. The other three normal cells were now rebuilt with like results. If changes in sensitivity occurred they are less than 5 per cent. and cannot be detected with the test method used. These six cells have now been in use for more than two months and have shown complete dependability. I have, so far, found no evidence that these particular Weston photocell discs were damaged by the direct exposure to the sun that they received. I am able to report, however, that certain electrocells, tested in conjunction with C. I. GUNNESS of Massachusetts State College, have shown that excessive exposure to sun has damaged the photocell discs. It seems highly inadvisable therefore to expose cells of this type to direct sunlight.

Through the courtesy of B. E. GILBERT of Rhode Island State College, I am able to report the results of tests, similar to those on dependability given above, which were run on some of his photocells. After finding the defective cells in my own unit I thought it would be interesting to run a like test on his

[3] These springs must not exert more than about a pound pressure for otherwise they may short the photocell disc.

multiple-celled photounit. These cells had never been exposed to direct sunlight. When the five cells were tested, however, one was found to be only 25 per cent. as strong as the other four. When it was wedged, it became the best cell of the five in the unit. Also one of the large Weston illuminometers used in the same laboratory increased its reading from 940 foot-candles for this same light to 1500 foot-candles. This occurred in response to pushing the glass face of the cell back and forth several times. No subsequent wedging or moving of the face would change the reading in the least. This latter effect has been observed very often during the progress of these studies. Perhaps this may be due to some oxidation which reduces the efficiency of the electrode contacts. There are other photocells of the voltaic type which seem to have few if any of the mechanical weaknesses which have been described above. One of these, an electrocell, has received more than two months' exposure under a sphere and has maintained perfect stability.

In one case, during these experiments, lightning struck nearby and burned out a Weston photocell. On examination, a tiny pit, about $\frac{1}{4}$ mm. across and about the same depth was observed on the face of the photocell disc. The surface of the disc was explored by laying the disc on one copper wire and touching a moistened wire to various points on the face. The pit already mentioned was the dead spot. A tiny bit of wax (half beeswax and half rosin) was put on the pit and a tiny bit of paper pushed down against it. The cell now functions perfectly and its calibration seems unchanged.

Integration of light records for various foot-candle levels

In view of the interest shown in a suggestion I made at the symposium I shall elaborate somewhat and give a figure to make the suggestion more concise and clear. It would seem from the data already available on the light intensities required for various processes, especially photosynthesis, that different species have different optima. If, then, one is working with a plant that reaches its maximum rate of photosynthesis at the 2000-foot-

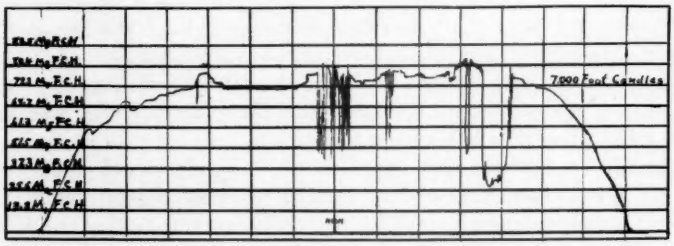

Fig. 10. Record for August 15, 1935, showing a method of integrating light records at various foot-candle levels for processes not requiring full sunlight intensity for their maximum rate. For full description see text.

candle level, then those portions of the light record lying above that level act only to obscure the true relationships. Might it therefore not be of value to integrate the records for various levels and give each investigator those values adapted to his needs?

In figure 10 is shown an ordinary record for Storrs, Connecticut, on August 15, 1935. It is calibrated in 1000-foot-candle levels. At the left of the figure are shown the integrated values obtained with a planimeter for these various plateaus. I have designated the first one as 13.8 M_1 f.c.h. This would read 13.8 foot-candle-hours of light below 1000-foot-candle level. The second level would be 25.6 M_2 f.c.h. or 25.6 foot-candle-hours below the 2000-foot-candle level, etc. It will be noted that each level is a complete summation of the total light quantity to that level so that finally when the level of maximum intensity for the day is reached the figure automatically becomes the total light quantity for the day. Thus the top plateau can be read at 80.5 M_9 f.c.h. or as 80.5 times M (1000) or 80,500 foot-candle-hours.

Summary

1. A comprehensive series of experiments on various physical arrangements of the sensitive surfaces of photocells were run to determine the combinations which gave the most perfect sampling of light. These different sampling devices were connected to a duplex recorder so that the sampling of the units was recorded as continuous, simultaneous curves of the same light station on the one-recorder drum.

2. Six types of photounits were tested. Of these the two found to have the least error in sampling were a six-celled unit in which a photocell was fastened on each of the six sides of a cube and the whole enclosed beneath a spherical integrating shade of opal glass, and a one-celled unit in which one photocell was placed in the base of an integrating sphere facing up. There was no significant difference between the sampling of these two units, but certain other considerations would indicate that the one-celled unit may be preferable.

3. The determination of visible radiation by exposure of the receiving unit horizontally to the direct rays of the sun, as is done by the Weather Bureau for total radiation, does not seem suited to physiological studies, owing to the impossibility of correcting by the cosine law for lateral light and other effects. Also the exposure of photocells to the direct rays of the sun may be very detrimental to them.

4. A suggestion is made for a method of integrating light records for various foot-candle levels to enable each investigator to choose those levels of significance in his studies.

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A PRECISE METHOD, WITH DETAILED CALIBRATION FOR THE DETERMINATION OF ABSORPTION COEFFICIENTS; THE QUANTITATIVE MEASUREMENT OF THE VISIBLE AND ULTRAVIOLET ABSORPTION SPECTRA OF ALPHA CAROTENE, BETA CAROTENE, AND LYCOPENE[1]

ELMER S. MILLER[2]

(WITH EIGHT FIGURES)

Introduction

Owing to recent developments in amplifying tubes and circuits, it has been possible to design a spectrophotoelectric apparatus which permits the precise measurements of the absorption coefficients of alpha carotene, beta carotene, and lycopene. The absorption curves are important as: (a) *a criterion in following the isolation of plant pigments* (**6**), and (b) *standards in analytical methods.*

Recently ZSCHEILE (**11**) and MILLER (**7**) have shown that the spectrophotoelectric method satisfactorily solves the problem of pigment differentiation. This method makes possible not only the determination of the total differentiation for each group (chlorophylls, carotenes, and xanthophylls), but also the analysis for the total of each component within the respective groups. Because no separation of pigments into groups or components within a group is necessary, the spectrophotoelectric method is rapid.

This paper presents a detailed calibration of the apparatus and a comprehensive discussion of the limitations of the method in conjunction with the quantitative absorption spectra of alpha carotene, beta carotene, and lycopene in the visible and ultraviolet region.

Nomenclature

The nomenclature employed in quantitative spectroscopy is somewhat confusing to biologists because different units and symbols are employed to express absorption. In this paper alpha (α) in equation 4 is plotted as the ordinate with wave lengths expressed in Ångstrom units as the abscissa.

The quantitative absorption spectrum of an organic compound is determined in the following manner. In a homogeneous medium, according to Lambert's law, the amount of radiant energy absorbed is expressed by equation 1.

$$I_x = I_o \ 10^{-\beta x} \qquad (1)$$

[1] Contributions from the Department of Botany, University of Minnesota.

[2] This research was started at the Herbert Jones Laboratory at the University of Chicago in 1932, and continued at the Department of Botany at the University of Minnesota from September, 1936. The absorption curves in the visible region for the mentioned carotenoids have been published in the *Botanical Gazette* and *Plant Physiology.*

When beta (β) is proportional to concentration, Beer's law is obeyed, and

$$\beta = \alpha c. \tag{2}$$

Then

$$\log_{10} \frac{I_o}{I_x} = \alpha c x \tag{3}$$

or

$$\alpha = \log_{10} \frac{I_o}{I_x} / cx. \tag{4}$$

I_o = intensity of light transmitted by the solvent cell.
I_x = " " " " " " solution cell.
x = thickness of absorption cell in centimeters.
c = concentration in grams or moles per liter.
α = specific absorption coefficient for grams per liter, or molecular absorption coefficient for moles per liter.

McNICHOLAS (**5**) plots "absorbency" as the ordinate. He defines this symbol as:

$$\text{"absorbency"} = \log_{10} \frac{I_o}{I_x} - bcK \tag{5}$$[3]

Since his units are the same as those employed in equation 4, $K = \alpha$. WEIGERT (**10**) employs E to express the molar absorption coefficient calculated as the logarithm to base 10. When the molecular weight is not known, α is frequently calculated on the basis of a 1 per cent. concentration. In this paper, unless stated, the gm.-cm. units will be employed.

Materials and methods

SPECTROPHOTOELECTRIC APPARATUS

The design of the apparatus is similar to the one described by ZSCHEILE (**12**), save that certain modifications have been made so that narrower slit-widths could be employed.

The spectral region is isolated from the continuous spectrum by a Zeiss fixed arm spectroscope (deviation 90°), used as a monochromator. Referring to wave length 4861 Å, the effective aperture is F/6.4 for the entire instrument. The error of the wave length drum is less than ± 1 Å at 2150 Å, and ± 3 Å at 7800 Å. Although the Zeiss instrument is equipped with achromatic lenses, a slight adjustment is necessary to correct for chromatic errors.

For the visible region, a 50 c.p. automobile Mazda bulb connected to batteries is employed as the light source. For the ultraviolet region a Urey type of hydrogen discharge tube operating at 4000 volts and 0.8 amp. is rather satisfactory.[4]

[3] b = cell length in centimeters.

[4] The tube was lined with a 5-mm. quartz tube. This precaution and the operation

A DuBridge and Brown (7) circuit (fig. 1) is employed to measure the photoelectric current. In this circuit an F.P. 54 vacuum tube (General

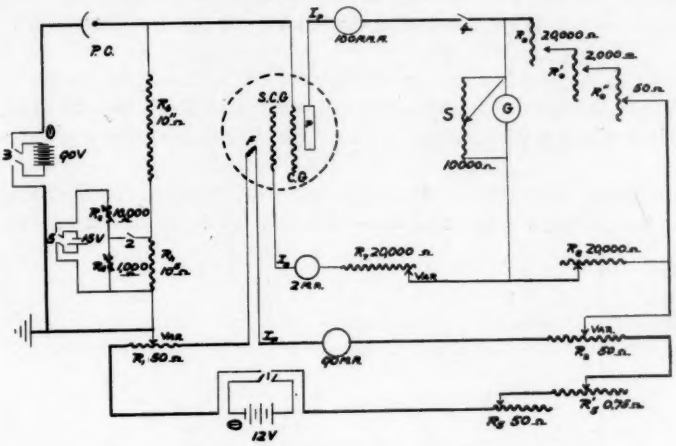

FIG. 1. A modified DuBridge d.c. amplifying circuit.

Electric) is employed for the amplification of the photoelectric current. The photoelectric current is passed through a high resistance Rg (1×10^{11} ohm), and the potential across this resistance is impressed on the grid of the amplification tube. The amplified current is measured by a galvanometer (Leeds Northrup Company: 2500-b, period 6 sec.). The sensitivity of the galvanometer is controlled by an Ayrton shunt. The photocell is a caesium oxide gas-filled type with internal and external grounded guard rings. The sensitivity curve of this cell has been described by Young and Pierce (13).

The entire apparatus was set up in an underground laboratory in which the temperature fluctuates less than $\pm 0.4°$ C. in 24 hours. With proper electromagnetic shielding, a stable voltage sensitivity of 225,000 mm. per volt and a current sensitivity of 1×10^{-16} amp. per mm. is attained. These sensitivities are for 4 meters scale-to-galvanometer distance and a galvanometer sensitivity of 0.6. The galvanometer drift is less than 0.5 cm. per hour with oscillations ± 1 mm. Usually 40 to 50 seconds are sufficient for the measurement of two absorption coefficients. The cell carriage, which holds three absorption cells (Hilger type 291), is placed behind slit no. 2. Thus from four transmissions, two absorption coefficients may be calculated.

The over-all error (difference between I_o' and I_o'') for the apparatus, including fluctuations in light source, amplification tube, photoelectric cell,

at 0.8 amp. prolongs the life of the tube greatly. The present tube has been in operation over ten months. It has run 500 hours on one filling with hydrogen.

and galvanometer, was less than $\pm$ 0.25 per cent. for the visible region and less than $\pm$ 0.35 per cent. for the ultraviolet region. The larger error for the ultraviolet region was due to fluctuations in the light source. This error should not be confused with the total error involved in alpha (α_T).

Calibration

The calibration of the apparatus is described in detail so as to familiarize other investigators with the method employed in obtaining these spectroscopic data.

The Cooper-Hewitt mercury vapor arc was employed for the calibration of the wave length drum and the collimator lens. Figure 2 contains two

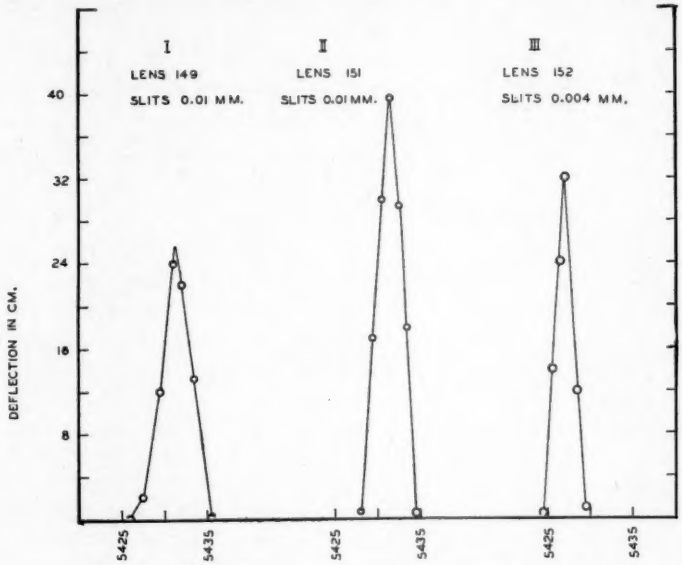

Fig. 2. Absorption curves of the 5460.7-Å mercury arc line.

absorption curves of the 5460.7-Å mercury line. After a preliminary attempt at setting the wave length drum and collimator lens, curve I was obtained. From this curve, with the wave length drum set at wave length 5432 Å, the collimator lens was again focused. These adjustments showed that the maximum galvanometer deflection was obtained with the collimator lens set at 150. Then curve I was repeated with readings taken at approximately one-Ångstrom intervals. These data are plotted in curve II. This procedure was again repeated and curve III was obtained. Thus, the 5460.7-Å line was in best focus with the collimator lens set at 150 and wave

length drum at 5432 Å. The agreement between curves I, II, and III is excellent, considering that the minimum error in the wave length drum setting is ± 0.5 Å. The maximum discrepancy between two wave length drum calibrations at six-month intervals for both the visible and the ultraviolet region was 2 Å.

The apparatus was tested to ascertain whether correct measurements could be obtained, according to the following procedure.

A. Known voltages were impressed on the grid of the amplification tube and the deflections of the galvanometer were observed. Direct proportionality was found.

B. The proportionality between the response of the galvanometer and the intensity of the incident light on the photoelectric cell was tested as follows:

1. With the deflections of the galvanometer 80–100 cm. (reduced to 35 cm. at wave length 2200 Å) the response was measured when a series of screens was interposed. These data are summarized in table I.

TABLE I

COMPARISON OF SCREEN TRANSMISSIONS MEASURED BY THERMOPILE AND PHOTOELECTRIC APPARATUS WITH ABSORPTION CELLS IN PLACE

APPARATUS	SCREEN TRANSMISSIONS		
	SCREEN NO. 1	SCREEN NO. 2	SCREEN NO. 3
Thermopile	73.4	54.8	32.7
2250 Å with cell no. 1	73.0	54.6	33.0
2250 Å with cell no. 2	73.3	54.4	33.0
2250 Å with cell no. 3	73.5	54.4	33.1
3000 Å with cell no. 1	73.0	54.6	32.5
3000 Å with cell no. 2	73.0	54.4	33.0
3000 Å with cell no. 3	73.3	54.4	32.9
3800 Å with cell no. 1	73.5	55.0	32.9
3800 Å with cell no. 2	73.6	54.7	32.4
3800 Å with cell no. 3	73.3	55.0	32.7
4000 Å with cell no. 1	73.8	54.4	32.4
4000 Å with cell no. 2	74.0	54.8	32.4
4000 Å with cell no. 3	74.0	54.3	32.2
5000 Å with cell no. 1	73.6	54.4	32.4
5000 Å with cell no. 2	73.8	54.8	32.2
5000 Å with cell no. 3	73.4	54.8	32.3
6000 Å with cell no. 1	73.7	54.5	32.3
6000 Å with cell no. 2	73.8	54.3	32.7
6000 Å with cell no. 3	73.7	54.3	33.0
7000 Å with cell no. 1	73.6	54.6	33.1
7000 Å with cell no. 2	73.7	54.4	32.8
7000 Å with cell no. 3	73.7	54.4	33.0

Because three absorption cells were employed in this study, the screens were interposed with each of the respective cells in the path of the beam of light at 1000-Å intervals. Previously, a similar test was made with only one cell and screen no. 2 at 100-Å intervals. The transmission values of the screens were measured with a thermopile. Owing to variations in the respective position of the screens, the error in this experiment is 1 per cent.

2. The apparatus was tested for the linearity of response to incident light intensities which gave 30-, 60-, and 120-cm. deflections of the galvanometer, respectively. This was primarily to check "fatigue effects" in the photocell and "capacity effects" in the amplifying circuits. When the screens were interposed, constant transmission ratios at the different light intensities were obtained.

C. Standard solution.—As a check on the total error of the apparatus, light source,[5] and observer, the absorption coefficients were measured on a standard solution for the visible and ultraviolet region. This solution consisted of equimolar concentrations of potassium chromate and copper sulphate (pentahydrate) dissolved in a two-normal

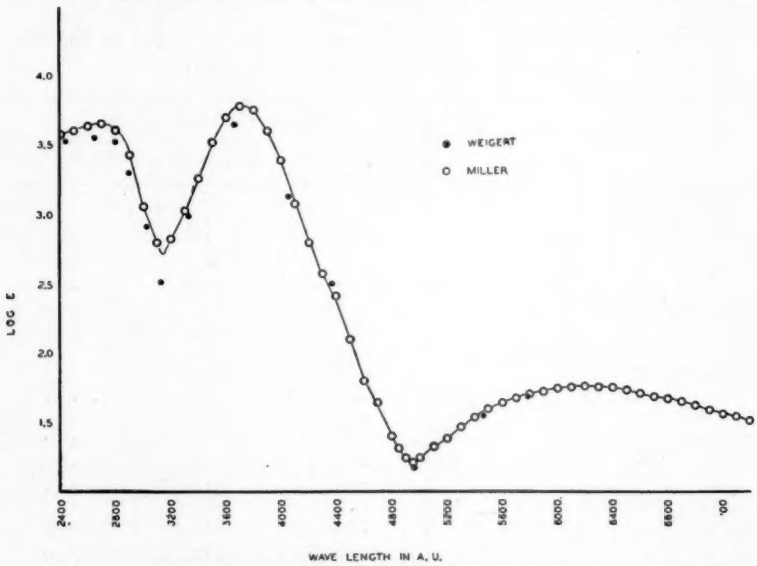

FIG. 3. Absorption spectra of equimolar concentrations of potassium chromate and copper sulphate (pentahydrate) in 2 N ammonia.

[5] Occasionally when the hydrogen discharge tube has been in service for some time, new bands are superimposed on the hydrogen lines. Some evidence has been obtained that the presence of these new bands may affect the value of the absorption coefficient.

ammonia solution. This standard was first employed by WEIGERT (**10**), VON HALBON (**2**), and recently by SMITH (**9**). The absorption spectrum of this standard is presented in figure 3. The dots and the circles represent the values obtained by WEIGERT (**10**) and the writer respectively. Considering the low sensitivity of WEIGERT's apparatus and the summation of the total errors in the two absorption curves, the agreement is about as good as may be expected. Unfortunately, this inorganic salt mixture has the following limitations as a standard:

1. The values of the absorption coefficients are affected by the normality of the ammonia solution.
2. For the visible region, the absorption bands are too broad for this mixture to be a good standard in quantitative absorption spectroscopy. This will become apparent in the forthcoming discussion under the heading "Slit data and scattering of light."

D. Calibrated glass filter.—Through the courtesy of the Bureau of Standards a calibrated filter was obtained. The transmission of this filter was measured with a thermopile at the wave lengths stated in table II.

TABLE II

COMPARISON OF TRANSMISSION VALUES OF GLASS FILTERS AS MEASURED BY THERMOPILE AND PHOTOELECTRIC APPARATUS

APPARATUS	TRANSMISSION VALUES		
	3020 Å	3130 Å	3340 Å
	%	%	%
Thermopile	29.5	54.5	86.0
Photoelectric	28.9 ± 0.2	54.6 ± 0.2	85.9 ± 0.1

The photoelectric data agree within the experimental error with the transmission values obtained with the thermopile. This is an excellent filter to check on the total error because of its steep slope. If the prism setting is more than ± 1.0 Å in error, the transmission error is increased by a factor of two.

SLIT DATA AND SCATTERING OF LIGHT

Before comparisons are made with data obtained by investigators in different laboratories, *i.e.*, the use of the absorption curves in quantitative analyses, it is essential to state:

1. Slit-width in millimeters actually employed at the respective wave lengths.

2. The effective dispersion, *i.e.*, the slit-width in terms of Å per mm. of slit-width to permit the calculation of the spectral region isolated.

The slit data for the photoelectric apparatus is presented in table III.

TABLE III

SLIT DATA FOR ULTRAVIOLET REGION (HYDROGEN DISCHARGE TUBE AT 0.8 AMP.) AND VISIBLE REGION (50 C.P. MAZDA LAMP) WITH A 2-CM. QUARTZ CELL FILLED WITH ETHANOL

WAVE LENGTH	REGION ISOLATED Å/MM. OF SLIT-WIDTH	SLIT-WIDTH	SPECTRAL REGION ISOLATED IN Å	DEFLECTION OF GALVANOMETER
Å	*Å/mm.*	*mm.*	*Å*	*cm.*
2200	65	0.20	13.0	19.5
2300	76	0.16	12.2	20.0*
2450	102	0.08	8.2	69.1
2600	132	0.04	5.3	62.9
2900	204	0.025	5.1	76.0
3450	388	0.022	8.5	65.6
4000 (Quartz)	630	0.022	13.8	60.8
4000 (Flint)	160	0.025	4.0	49.4
4200	200	0.016	3.2	59.0
4500	240	0.007	1.7	99.0
4800	302	0.0033	1.0	90.7
6000	680	0.002	1.35	120.0
7200	1400	0.002	2.8	132.8

The limits of the wave lengths emitted from the second slit is two times the slit-widths due to overlapping of rectangles of light (finite spectral regions isolated) emitted from slit number II. Hence, the base of the frequency histogram of wave lengths is twice the respective slit-width. In this case the distribution curve is a triangle rather than one which approaches the normal curve. Therefore the population within the center half of the histogram (actual slit-width employed) includes 75 per cent. of the total wave lengths emitted. Data presented in table III show that in the visible and ultraviolet region, 75 per cent. of the total wave lengths emitted from the second slit are between 1.0 and 4.0 Å, and 5.1 and 13.8 Å for the visible and ultraviolet regions respectively.

SMITH (9) employed 0.07-mm. slit-widths which gave a spectral region 8.0 Å wide for the center half of the absorption curve of the 5460.7-Å line. This does not definie the spectral region isolated because the width of the mercury line is not only affected by the slit-widths but also by the type of mercury arc, voltage, and amperes employed, and distance of arc from slit no. I.

Heretofore, researches employing quantitative spectroscopy have been made, and in many instances the slit-widths employed have not been stated. Figure 4 contains the absorption curves for beta carotene with different slit-widths. At wave length 4520 Å the effect of opening the slits from 0.007 to 0.08 mm. was to increase the limits of the wave lengths emitted from the second slit from 1.7 to 19.5 Å. (This spectral region includes 75 per cent. of the wave lengths.) This decreased the value of alpha 10 per cent.

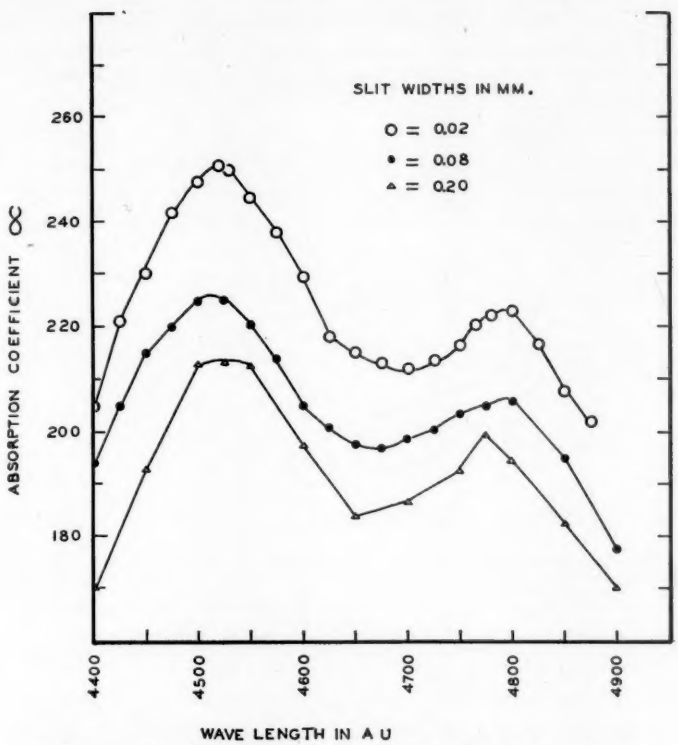

FIG. 4. Absorption spectra of β carotene at different slit-widths.

Figure 5 consists of curves representing the lowering of alpha, beta carotene, and lycopene by increasing the slit-widths. Similar curves were obtained when the mercury arc was employed as the light source. For the Zeiss monochromator, it would be useless to employ the standards presented in this paper with slit-widths wider than 0.05 mm. for quantitative analyses of the components within a group—even for estimating the total concentration for a group.

The value of alpha for compounds like benzene is affected more by slit-widths than those compounds with broad bands like the carotenoids and the inorganic standard presented in figure 3.

The simple method of determining whether "scattered" light (due to too wide slits) is of any importance is to replace the ordinary source of light by a monochromatic one, *i.e.*, mercury or sodium arc, without changing any of the other adjustments. This precaution should always be observed before quantitative measurements or analyses are made.

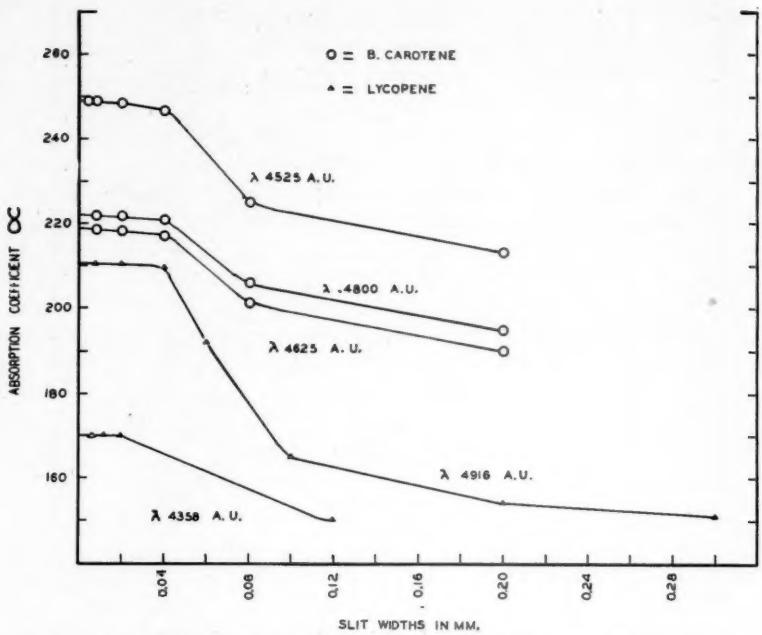

FIG. 5. The lowering of α at different wave lengths with increasing slit-widths.

After the proper calibration has been made to check the linearity between the incident light on the photocell and the deflections of the galvanometer, another test for scattered light is to measure alpha under conditions which give small and large values for I_o and I_x respectively—within concentration limits that obey Beer's law. If scattered light is a factor, the alpha, in which I_x is small, will be low. That is, a constant error, such as due to scattered light, will be increased with a small I_x. Another reason for employing very narrow slit-widths is to reduce the rate of photochemical reactions to a minimum.

Experimentation

PHOTOCHEMICAL DECOMPOSITION

In measuring absorption spectra of the common carotenoids, it is impossible in the ultraviolet region to eliminate photochemical reactions. In this study, the apparatus was so designed that by employing narrow slit-widths, it was hoped that the amount of photochemical decomposition could be decreased to less than the experimental error. At first, studies were made on the more stable carotenoids—alpha and beta carotene. In eight different

measurements of the absorption spectra no photochemical decomposition could be detected after irradiating the respective solutions 30 to 40 times in the course of determining the absorption spectra.

The next compound studied was lycopene. At the beginning of an experiment, alpha (α) was measured at wave length 3000 Å. After twenty subsequent measurements were made at 50-Å intervals throughout the entire ultraviolet region, alpha was again measured at wave length 3000 Å. This value of alpha checked with the first measurement. The error was 0.5 per cent. These experiments show that if very narrow slit-widths are employed, the amount of photochemical decomposition is insignificant, even for compounds as labile as lycopene.

The solvents employed in this investigation were purified as described by MILLER (**6**).

ERRORS

The inherent errors of the circuit have already been discussed in connection with the description of the amplification circuit.

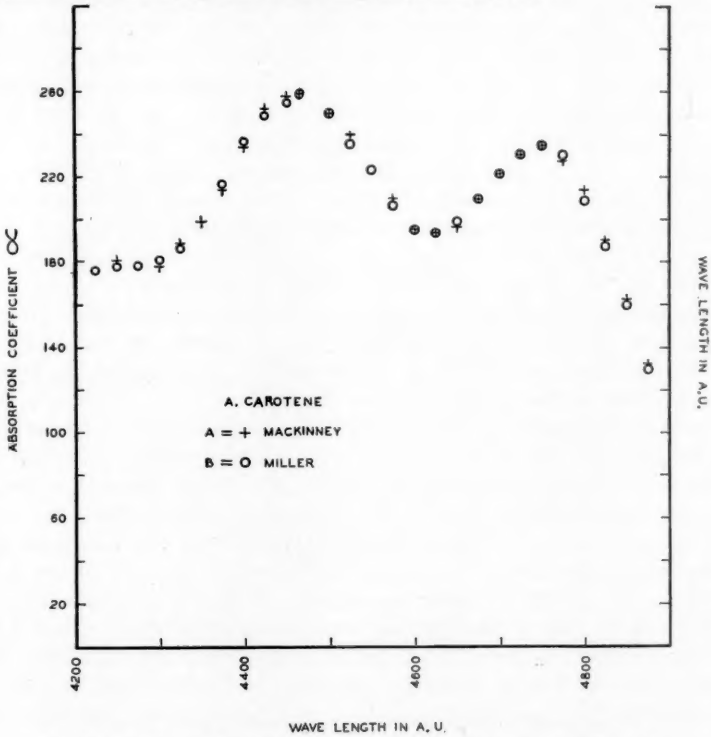

FIG. 6. Duplicate absorption curves measured by different manipulators.

A common criterion frequently employed in the purification of organic compounds is to regard the preparation with the largest absorption coefficient as being the purest. In the visible region, the discrepancy between purified preparations should be less than 1 per cent. In those regions where the absorption coefficient is less than 20 to 40, the difference between two samples may be 2 to 10 per cent. This increase in error is largely due to traces of impurities in the solvent and sample.

In the ultraviolet region the error is usually increased due to difficulties in keeping the absorption cells clean. Only in part can this factor be corrected. In this study the maximum error introduced by adsorption on the cell surfaces was 0.5 per cent. Likewise, the concentration range over which Beer's law is obeyed becomes more limited in the extreme ultraviolet. By employing different concentrations in the absorption cells, the concentration range over which Beer's law is obeyed is readily established.

Fortunately, the errors introduced by the apparatus, weighing, manipulators, balance, zero transmission of the absorption cells, dilutions, slit-width settings, and prism settings tend to cancel each other. In figure 6 this is illustrated by absorption curves A and B. In this experiment, two manipulators, working independently, measured the absorption of alpha carotene in the visible region. In view of the fact that the apparatus error was approximately $\pm$ 0.4 per cent. when this test was made, the agreement between the two sets of data is good. It is interesting to note that the largest discrepancies occurred at wave lengths 4350 and 4800 Å. At these wave lengths the slope of the absorption curve is large, and consequently a slight error in the prism settings would introduce a significant error.

Absorption spectra

The absorption spectra of alpha carotene, beta carotene, and lycopene have been quantitatively measured between wave lengths 2200 and 5400 Å. The absorption curves are presented in figure 7.[6] In this study, the precautions described by Miller (**6**) have been employed.

In the carotenoid literature the largest discrepancies occur between absorption spectra data obtained by investigators in different laboratories. The absorption coefficients of the carotenes and lycopene were first measured by Kuhn (**3**) and Smakula (**8**). When these data were published, no calibration data were presented. Miller (**6**) and Smith (**9**) were unable to confirm the values obtained by Kuhn and Smakula, although Smith's (**9**) curves agreed with those curves previously reported by Miller within the experimental error. Smith (**9**), in a detailed study concerning sources of errors, was unable to satisfactorily explain the discrepancies in absorption spectra data reported by Kuhn and Smakula, and Miller and Smith.

[6] In figure 7, the experimental values are presented, usually at 50-Å intervals because the graph was greatly reduced for publication.

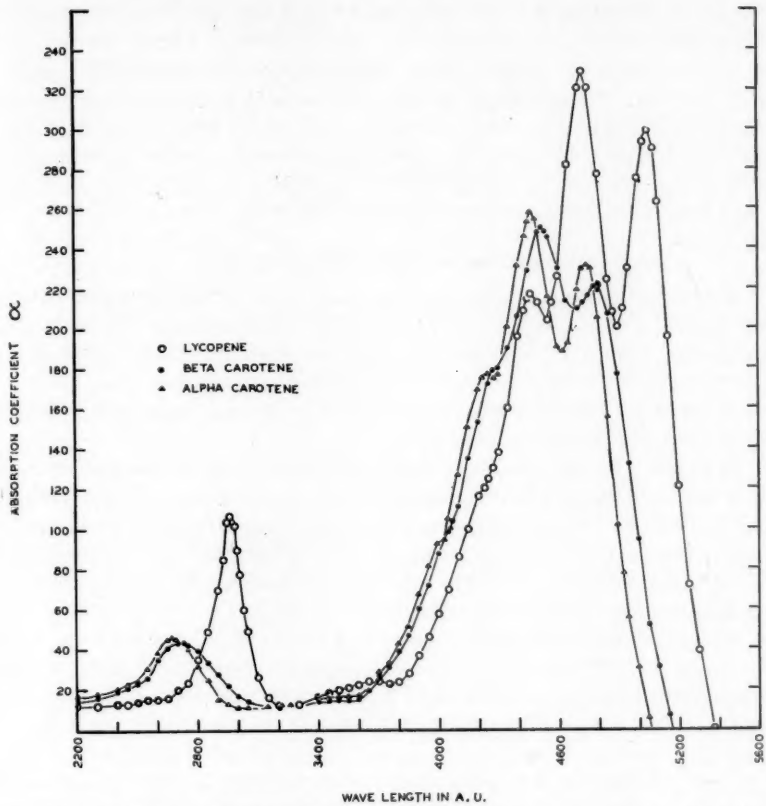

FIG. 7. Absorption spectra of α carotene, β carotene, and lycopene in 80 per cent. ethanol and 20 per cent. diethyl ether.

Readings were taken at 25-Å intervals, save 10-Å intervals over the maxima and minima.[6] The absorption spectrum of alpha carotene consists of four bands and a shelf. The maxima for the bands occurs at 4750 Å ($\alpha = 231.5 \pm 1.5$), 4465 Å ($\alpha = 258 \pm 1.5$), 4246 Å ($\alpha = 178 \pm 1.6$), and 2675 Å ($\alpha = 47.0 \pm 0.5$). The shelf occurs at 4010 Å. In the visible region a minimum occurs at 4625 Å ($\alpha = 190.0 \pm 1$). The extremely wide minimum in the ultraviolet (approximately the same for the carotenoids investigated in this study) will be discussed under oxidation.

The absorption curve for beta carotene has three definite maxima at wave lengths 4800 Å ($\alpha = 222 \pm 1.0$), 4525 Å ($\alpha = 249 \pm 1.4$), and 2790 Å ($\alpha = 43.0 \pm 0.5$). An apparent point of inflection occurs at 4055 Å and a

distinct shelf at 4300 Å ($\alpha = 180.0 \pm 1.0$). A minimum is observed at 4700 Å ($\alpha = 210.0 \pm 1.0$).

The lycopene was isolated from tomatoes, and furnished by Dr. M. B. MATLACK (**4**). Lycopene has four bands with well-defined maxima at wave lengths 5050 Å ($\alpha = 298 \pm 2$), 4725 Å ($\alpha = 328 \pm 2$), 4465 Å ($\alpha = 217 \pm 1$), and 2960 Å ($\alpha = 102.5 \pm 2$). In the visible spectrum, minima were found at 4900 Å ($\alpha = 201.5 \pm 2$) and at 4550 Å ($\alpha = 204 \pm 2$). A small shelf was noted between wave lengths 4200 and 4250 Å.

OXIDATION OF CAROTENOID SOLUTIONS

A detailed spectroscopic study has been made by MCNICHOLAS (**5**) on the oxidation of carotene (chiefly beta) and "leaf xanthophyll" in 80 per cent. ethanol and 20 per cent. ether. MCNICHOLAS reports that during the first "stages" of oxidation, there is a marked increase in a band at wave length 3410 Å. He concludes that his purest carotene samples exhibited a slight inflection at this same wave length.

In figure 7 the carotene curves also show a very small inflection between wave lengths 3400 and 3500 Å, and a similar inflection for lycopene at wave length 3700 Å. For the carotenes, this inflection is no greater than the experimental error; hence insignificant, save that many of the earlier studies report a distinct band. Therefore a few experiments were made to observe the rate at which these bands develop.

After the absorption coefficients of beta carotene and lycopene had been measured (fig. 7) the rate of oxidation was followed spectroscopically by the development of bands between wave lengths 3400 and 3800 Å. When the values of the alphas at the respective maxima had increased twofold, the absorption curve for beta carotene and lycopene were measured. These data are presented in figure 8. Curve B shows that on the fifth day after the carotene was dissolved in the solvent, the value of the absorption coefficients at wave length 3400 Å had increased twofold. On the second day, the value of alpha (curve E) for lycopene had increased from 20 to 43 at wave length 3650 Å.

Additional experiments were made to determine the rate of oxidation of the lycopene solution when the absorption cells were placed in front of slit no. 1, so that the lycopene solution was exposed to the total irradiation of the discharge tube. When the lycopene solution had been exposed less than 20 minutes, the value of alpha at wave length 3600 Å was larger than the absorption coefficient at wave length 2960 Å.

SIGNIFICANCE OF OXIDATION EXPERIMENTS

CAROTENES.—KUHN (**3**) and SMAKULA (**8**) in their spectroscopic investigations of the carotenes failed to observe an inflection in the absorption

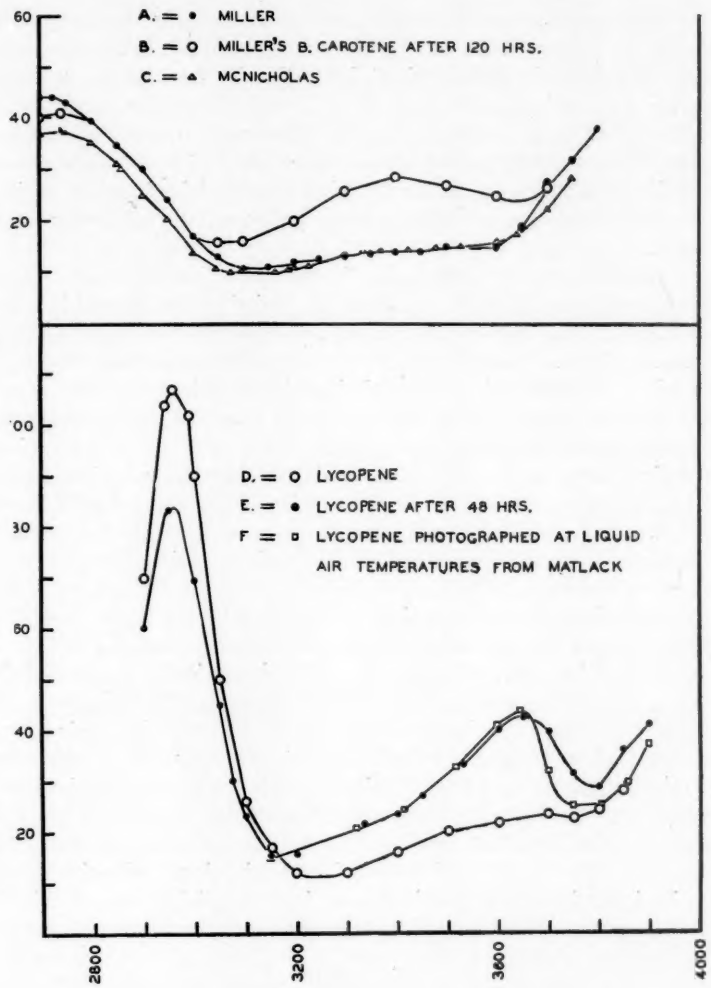

FIG. 8. Changes in β carotene and lycopene spectra accompanying oxidation.

curve of either alpha or beta carotene in hexane. KUHN's carbon and hydrogen analyses demonstrated that the respective carotene samples were pure. He also employed a hydrocarbon as solvent, which would cause a slower rate of oxidation than a mixture of ethanol and ether.

Since KUHN employed aluminum oxide (Fasertonerde) in his method of purifying the carotenes, the 3400-Å inflection (band) is not due to oxidative changes introduced by the adsorbent, but rather by the solvent, oxygen, and light.

The small inflection observed by MCNICHOLAS (5) no doubt is due to impurities, because the value of alpha at wave length 4250 Å for his carotene sample is 1.5 to 2 per cent. lower than that presented in figure 7. This discrepancy is of the same magnitude as that presented by his increase at wave length 3400 Å.

LYCOPENE.—For some tme it has been known that lycopene is readily oxidized, even more so than the carotenes. This is demonstrated in a study by SMAKULA (8). He measured the absorption curve in hexane and, although he found a distinct band at wave length 3650 Å, he does not record it as such. MATLACK (4) succeeded in isolating pure lycopene from tomatoes —purity based on carbon and hydrogen analyses. His associates measured the absorption coefficients of lycopene in visible and ultraviolet region at liquid air temperatures. MATLACK obtained evidence that a lycopene dissolved in ethanol and ether was partially oxidized after standing 12 hours in the dark. The data in figure 7 confirm the absorption curve reported by MATLACK for the visible region. In the ultraviolet region MATLACK reports bands at wave lengths 3650 and 2960 Å.

After the lycopene curve in figure 7 was measured, the absorption coefficients were again determined on 48-hour-old lycopene solutions. These data are presented in figure 8, curve E. The absorption curve reported by MATLACK and SANDO was measured photographically and is, in part, represented by curve F.[7] It is interesting to note that even at liquid air temperatures and a time interval as short as that necessary for a spectrogram, approximately as much photooxidation occurred as that (oxidation) occurring when a similar solution is allowed to stand 2 days at 20° C. These data suggest that the band (or inflection) at wave length 3650 Å, as reported by SMAKULA, MATLACK, and SANDO, is due to oxidation. Likewise the small inflection in the lycopene curve in figure 7 is due to slight traces of oxidation-inflection approximately that of the experimental error.[8]

Summary

1. A precise method has been developed for the quantitative measurement of absorption coefficients. Data are presented to show that for carotenoid research the absorption cells must be placed behind slit no. 2. When

[7] The curve is reproduced as accurately as it is possible to read the values from figure 4 in reference (4).

[8] While this paper was in press, a paper by HOGNESS, ZSCHEILE, and SIDWELL has been published in the March, 1937 issue of *Physical Chemistry*. This paper concerns the construction, calibration, and the use of a photoelectric spectrophotometer in chemistry.

this precaution is observed and narrow spectral regions are employed, the photooxidation is so decreased that, during the measurement of an absorption curve, the total decomposition is less than the experimental error.

2. A procedure for a detailed calibration of the photoelectric apparatus has been discussed. Suggestions are made which will make it easier for investigators in different laboratories to obtain concordant results.

3. Calibrated filters obtained from the Bureau of Standards are the best single test to ascertain that the apparatus will give correct measurements.

4. Precautions and limitations are discussed as to when it is not possible to employ the standards, presented in this paper, for quantitative analysis.

5. The sources of errors are discussed. When the same apparatus is employed by several investigators, many of the possible errors cancel each other. The magnitude of the total errors in the various parts of the visible and ultraviolet region has been stated.

6. The absorption coefficients in the visible and ultraviolet region have been measured in 80 per cent. ethanol and 20 per cent. ether for alpha carotene, beta carotene, and lycopene.

7. Data have been presented showing that a band at wave length 3400 Å, or even an inflection, is due to traces of carotene oxidation products present.

8. Photo- or chemical-oxidation or both are responsible for the absorption band at wave length 3650 Å, as previously reported for lycopene.

9. In the ultraviolet region, even at liquid air temperatures, it is impossible to obtain an accurate spectrogram of lycopene.

10. The bearing of the data presented in this paper, to that reported by other investigators has been discussed.

The writer wishes to express his appreciation in particular to Professor T. R. Hogness, under whose direction the visible absorption studies were made; to Dr. F. P. Zscheile for aid received throughout the entire study; to the Department of Botany at the University of Chicago for plants needed in the carotenoid preparations; to Dr. Guy McKinney for data presented in figure 6; to the Board on National Research Fellowships in the Biological Sciences for making the first part of this study possible by the grant of a fellowship; and to Professors C. O. Rosendahl and Geo. O. Burr, both of whom have given constructive criticism and have made possible the presentation of this paper. The writer wishes to express his appreciation to the Rockefeller Foundation, New York, for making possible the use of the equipment at the University of Chicago; to Dr. M. B. Matlack, Bureau of Plant Industry, for the lycopene; and to the members of the Bureau of Standards for the excellent calibrated filter.

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ENZYME ACTIVITY IN COLD-HARDENED AND UNHARDENED RED CLOVER[1]

GLENN A. GREATHOUSE AND NEIL W. STUART

Introduction

The ultimate objective of winter-hardiness investigations is to find the fundamental nature of this phenomenon. Toward that end results are reported of a study on the autolysis of red clover root and crown tissue when in the cold-hardened and unhardened condition. Results are also reported from experiments on hardened red clover plants stored for 11 days at temperatures of 34°, 31° and 12° F. Carbohydrate and nitrogen analyses were made on these tissues. It has been suggested (**8, 9**) that the sugars are related to protein precipitation, thus a study of the activity of the proteolytic and carbohydrate enzymes under similar conditions would appear to be of great interest and value.

Literature review

An extended review of the literature on various enzymes is outside the scope of this paper. The most recent work on enzymes as related to winter-hardiness is that of TYSDAL (**11**) on diastatic activity in alfalfa plants. NEWTON and BROWN (**7**) and others have noted a relation of winter-hardiness and catalase activity, the hardier variety having the higher activity.

The importance of sugars in the hardening process has been suggested by LIDFORSS (**5**), AKERMAN (**1**), NEWTON and BROWN (**8**), SCHAFFNIT (**9**) and others. Higher concentrations of sugars are associated with winter-hardiness in clovers (**2, 3**) and the question immediately arises, what changes occur within the plant to promote and retain the increase of sugars? TYSDAL (**11**) studied the diastatic power of the alfalfa plant as related to the hardened-off condition. In his investigation only the diastatic enzymes were studied. This still leaves the question unanswered as to whether the increase in sugars during the hardening period is due to conversion of starch to sugar, or whether the increase in sugar is not at the expense of starch, as TUMANOW (**10**) and others have suggested. Or, again, it may be that the hardier variety is more conservative of its already formed sugar, possibly because of a lower respiration rate.

KLING (**4**) reported that winter cereals could be differentiated as to cold hardiness on the basis of their protein ferments.

[1] Contribution from the University of Maryland, College Park, Maryland.

Materials and methods

The Ohio and French varieties of red clover[2] were selected as types that are winter hardy and less hardy respectively. The plant material for the autolysis experiment was grown on experimental plots which were seeded on April 21, 1934, on the Maryland Experiment Station Farm at College Park, Maryland. A 2–12–4 fertilizer was applied at the rate of 600 lb. per acre to the sandy loam soil previous to seeding. The seed was inoculated and planted without a companion crop at the rate of 15 lb. per acre. The plants were clipped at a height of 4 in. on September 12, 1934.

On January 30, 1935, both varieties of clover plants were removed from the snow-covered ground and separated into roots and crowns. All of the living shoot tissue was included with the crowns. The tissue was prepared for sampling as previously described (**2**). Suitable portions of the macerated tissue were placed in Kohlrausch or Erlenmeyer flasks, thoroughly wetted with toluene-saturated water, plugged with cotton, and placed in an oven at 37 ± 0.2° C. One set of the samples was analyzed immediately and others after autolysis periods of 24, 48 or 120 hours. An additional set of samples was placed in a water bath at 70° C. for 1 hour and then transferred to the 37° C. oven for 24 hours. Another set of samples was frozen at −22° C. for 1 hour and then placed at 37° C. for 24 hours. Care was taken that the samples were saturated with toulene water at all times during the autolysis at 37° C. The experiment was repeated on March 25, 1935, the only change being that some of the autolysis periods were shortened. At this time the plants were in the early spring stage just starting to grow.

The same varieties of clovers were seeded in rows in cypress boxes on October 14, 1935, and grew in the greenhouse until January 29, 1936. On this date the boxes containing the clover plants were placed in a cold storage room[3] which maintained a temperature of 34 ± 2° F. These plants received continuous light from four 500-watt Mazda lamps, placed at a distance of 3 feet from the plants. On February 11, 1936, the plants were divided into four uniform lots of 4 boxes each. Lot 1 remained at 34° F. and lots 2 and 3[4] were placed at 31 ± 1° F., and 12 ± 1° F., respectively. Lot 4 was taken for analysis on February 11 and designated as check in the tables. Lots 1, 2, and 3 did not receive illumination during the 11 days of storage as all storage rooms were dark.

Experimental results

The results of the carbohydrate analyses are shown in tables I to VI.

[2] Ohio F.C.I. Accession no. 20195; French, Accession no. 20141. The letters F.C.I. Indicate the accession number of the Division of Forage Crops and Diseases, Bureau of Plant Industry, United States Department of Agriculture.

[3] Through the courtesy of the Department of Horticulture, University of Maryland.

[4] Cold storage room, Arlington Farm, Va., through the courtesy of the Division of Fruit and Vegetable Crops and Diseases, Bureau of Plant Industry.

In the January autolysis experiment the invertase activity was greater in the Ohio roots than in the French roots. The data show that the starch and dextrin content of the Ohio roots decreased 30.2 per cent., while the French roots decreased 41.5 per cent. during the first 24 hours of autolysis, as measured by the percentage decrease over the original or check analysis (table III). The Ohio and French crown tissue decreased 63.5 per cent. and 94.9 per cent. respectively, in the starch and dextrin fraction over the same period of time. Heating the tissue in January to 70° C. for 1 hour did not destroy the carbohydrate enzymes, although accumulation of reducing sugars was not quite so great as in the untreated samples. The enzymatic activity for the starch and dextrin fraction was stimulated in all samples except French crowns by heating to 70° C. for 1 hour previous to the 24 hours of autolysis. The data indicate that the carbohydrate enzymes in the French tissue were injured more than those in the Ohio tissue by the heat treatment.

TABLE I

DISTRIBUTION OF CARBOHYDRATES IN RED CLOVER ROOTS AND CROWNS SAMPLED JANUARY 30, 1935. RESULTS ARE EXPRESSED AS PERCENTAGE OF THE DRY WEIGHT

PERIOD OF AUTOLYSIS	REDUCING SUGARS	SUCROSE	TOTAL SUGARS	STARCH AND DEXTRINS	HEMICELLULOSE
hours	%	%	%	%	%
	OHIO ROOTS				
(Check) 0	2.06	10.65	13.40	13.55	8.89
24	19.13	0.0	19.34	9.32	6.78
1 hr. 70° C.; 24 hr. 37° C.	18.86	0.69	20.25	4.82	7.93
1 hr. –22° C.; 24 hr. 37° C.	18.84	1.27	20.84	9.81	7.31
48	21.05	0.23	22.12	9.66	6.27
	FRENCH ROOTS				
(Check) 0	1.92	8.40	10.86	8.81	9.86
24	16.07	0.24	16.93	5.16	8.94
1 hr. 70° C.; 24 hr. 37° C.	15.02	0.65	16.25	4.35	8.94
1 hr. –22° C.; 24 hr. 37° C.	15.49	0.39	16.48	5.07	8.66
48	18.67	0.96	20.60	5.42	7.86
	OHIO CROWNS				
(Check) 0	4.02	6.33	10.88	4.47	9.36
24	14.53	0.18	15.30	1.63	8.21
1 hr. 70° C.; 24 hr. 37° C.	12.55	0.02	13.05	1.37	9.24
1 hr. –22° C.; 24 hr. 37° C.	14.65	0.0	14.53	1.80	7.73
48	15.42	0.63	16.79	1.57	6.72
	FRENCH CROWNS				
(Check) 0	3.20	5.15	8.78	2.30	10.25
24	11.70	0.42	12.41	0.12	9.64
1 hr. 70° C.; 24 hr. 37° C.	9.54	0.12	10.11	0.92	10.51
1 hr. 22° C.; 24 hr. 37° C.	12.07	0.28	12.87	0.15	9.05
48	12.38	0.18	13.22	0.11	7.93

Freezing the tissue at – 22° C. for 1 hour previous to autolysis had little, if any, effect upon the carbohydrate enzymatic activity (tables I, II).

TABLE II

DISTRIBUTION OF CARBOHYDRATES IN RED CLOVER ROOTS AND CROWNS SAMPLED MARCH 25, 1935. RESULTS ARE EXPRESSED AS PERCENTAGE OF DRY WEIGHT

PERIOD OF AUTOLYSIS	REDUCING SUGARS	SUCROSE	TOTAL SUGARS	STARCH AND DEXTRINS	HEMICELLULOSE
hours	%	%	%	%	%
			OHIO ROOTS		
(Check) 0	2.33	2.54	5.22	3.95	11.82
6	7.05	0.0	7.34	2.12	9.78
24	9.37	0.34	10.10	1.65	8.61
1 hr. 70° C.; 24 hr. 37° C.	8.09	0.0	8.43	1.49	10.47
			FRENCH ROOTS		
(Check) 0	1.74	0.59	2.52	1.88	12.23
6	4.23	0.0	4.28	0.79	10.21
24	5.23	0.40	6.05	0.76	8.87
1 hr. 70° C.; 24 hr. 37° C.	3.41	0.0	3.59	1.01	11.08
			OHIO CROWNS		
(Check) 0	3.35	0.13	3.20	2.33	11.41
6	4.92	0.0	5.03	1.02	7.64
24	6.66	0.13	7.08	0.92	7.46
1 hr. 70° C.; 24 hr. 37° C.	4.02	0.0	4.25	1.07	10.17
			FRENCH CROWNS		
(Check) 0	2.23	0.16	2.56	2.06	9.89
6	3.69	0.0	3.91	0.84	7.66
24	5.76	0.06	6.11	0.77	6.58
1 hr. 70° C.; 24 hr. 37° C.	2.31	0.0	2.49	1.68	9.24

In March, after growth had started, the clover roots and crowns were lower in total sugars, starch and dextrins than in January. The autolysis periods for March were 6 and 24 hours, respectively, in contrast to 24 and 48 hours for January. After 24 hours of autolysis on the tissues sampled in March the differences between the varieties tended to disappear. As in January, greater increases in reducing sugars were observed in the Ohio roots than in the French roots upon autolysis. Conversely the starch and dextrin fraction decreased more in the French roots than in the Ohio roots. Similarly to the January sample the reducing sugar increase and the starch and dextrin decrease were proportionally greater in the French crowns, although the differences were not as great for the March analysis. Following 6 hours of autolysis the starch and dextrin fraction had decreased 46.3 per cent. in the Ohio roots and 58.1 per cent. in the French roots. Decrease in the starch

and dextrin fraction of the Ohio and French crowns of 56.5 per cent. and 59.2 per cent., respectively, was noted over a similar period of autolysis. Heating the tissue sampled in March to 70° C. for 1 hour previous to autolysis decreased the enzymatic activity in all cases, except for the starch and dextrin hydrolysis in the Ohio root tissue. There was apparently less protection of the enzymes in March than in January. The protective action was very poor in the French crown tissue in both January and March. Data from tables I, II, VII, and IX show that there was about twice as much total sugar, amino acids, etc., in both varieties in January as in March.

TABLE III

DISTRIBUTION OF CARBOHYDRATES IN RED CLOVER ROOTS AND CROWNS SAMPLED JANUARY 30, 1935. RESULTS ARE EXPRESSED AS PERCENTAGE OF THE ORIGINAL, TAKEN AS 100 PER CENT.

PERIOD OF AUTOLYSIS	REDUCING SUGARS	SUCROSE	TOTAL SUGARS	STARCH AND DEXTRINS	HEMICELLULOSE
hours	%	%	%	%	%
			OHIO ROOTS		
24	927.0	0.0	144.3	69.8	76.3
1 hr. 70° C.; 24 hr. 37° C.	914.0	6.5	151.5	36.1	89.2
1 hr. −22° C.; 24 hr. 37° C.	913.2	11.9	155.5	73.4	82.2
48	1020.5	2.2	165.1	72.3	70.5
			FRENCH ROOTS		
24	837.3	2.9	155.9	58.5	90.7
1 hr. −70° C.; 24 hr. 37° C.	782.4	7.7	149.7	49.4	90.6
1 hr. −22° C.; 24 hr. 37° C.	807.3	4.7	151.8	57.5	87.8
48	973.0	11.4	189.7	61.5	79.6
			OHIO CROWNS		
24	361.3	2.7	140.6	36.5	87.6
1 hr. 70° C.; 24 hr. 37° C.	312.1	0.3	119.9	30.7	98.7
1 hr. −22° C.; 24 hr. 37° C.	364.4	0.0	133.5	40.2	82.6
48	383.8	9.9	154.2	35.1	71.7
			FRENCH CROWNS		
24	365.8	8.1	141.3	5.1	93.9
1 hr. 70° C.; 24 hr. 37° C.	298.4	2.3	115.1	40.1	101.0
1 hr. −22° C.; 24 hr. 37° C.	337.4	5.5	146.6	6.4	88.3
48	387.2	3.6	150.5	4.6	77.4

A comparison of relative enzymatic activity in January and in March can be noted in tables III and IV. It is not possible to compare the increases in reducing sugars at the two periods, since the amount of total sugars, starch, etc., was not the same at these dates. However, the percentage of decrease in the starch and dextrin fraction is of interest and indicates the enzymatic

TABLE IV

DISTRIBUTION OF CARBOHYDRATES IN RED CLOVER ROOTS AND CROWNS SAMPLED MARCH 25, 1935. RESULTS ARE EXPRESSED AS PERCENTAGE OF THE ORIGINAL, TAKEN AS 100 PER CENT.

PERIOD OF AUTOLYSIS	REDUCING SUGARS	SUCROSE	TOTAL SUGARS	STARCH AND DEXTRINS	HEMICELLULOSE
hours	%	%	%	%	%
			OHIO ROOTS		
6	302.5	0.0	140.7	53.7	82.8
24	401.8	1.3	193.5	41.7	72.8
1 hr. 70° C.; 24 hr. 37° C.	347.1	0.0	161.7	37.7	88.5
			FRENCH ROOTS		
6	242.4	0.0	169.9	41.9	83.4
24	299.8	0.7	240.1	40.4	72.5
1 hr. 70° C.; 24 hr. 37° C.	195.5	0.0	142.5	53.7	90.5
			OHIO CROWNS		
6	146.6	0.0	157.1	43.5	66.9
24	198.5	1.1	220.9	39.3	65.4
1 hr. 70° C.; 24 hr. 37° C.	119.9	0.0	132.7	46.0	89.1
			FRENCH CROWNS		
6	165.7	0.0	152.7	40.8	77.4
24	258.9	0.4	238.7	37.2	66.5
1 hr. 70° C.; 24 hr. 37° C.	103.5	0.0	99.1	81.3	93.3

activity on this fraction at the two periods. The percentage of decrease in starch and dextrin in the Ohio root for January was 31.2 per cent. and in March 46.3 per cent. Similarly in the French root the starch and dextrin fraction decreased 41.5 per cent. in January and 58.1 per cent. in March. An autolysis period of 24 hours in March produced hydrolysis of about 60 per cent. of the starch and dextrin in both Ohio and French roots.

In January 63.5 per cent. of the starch and dextrins in the Ohio crowns were hydrolyzed in 24 hours. For the same period 94.9 per cent. of starch and dextrins in the French crowns were hydrolyzed. An autolysis period of 24 hours in March brought about hydrolysis of nearly 62 per cent. of the starch and dextrins in both Ohio and French crowns.

The distribution of the carbohydrates in red clover roots stored for 11 days in darkness at low temperatures is shown in table V. These plants were undisturbed until sampled for analysis. At all storage temperatures the Ohio roots maintained a larger percentage of total sugars, starch, and dextrins than did the French roots. The reverse relationship exists for the pentosan and hemicellulose fractions of these tissues. The apparent per-

TABLE V

DISTRIBUTION OF CARBOHYDRATES IN RED CLOVER ROOTS. RESULTS ARE EXPRESSED AS PERCENTAGE OF THE DRY WEIGHT

SAMPLING DATE	VARIETY	STORAGE TEMPERATURE	REDUCING SUGARS	SUCROSE	TOTAL SUGARS	STARCH AND DEXTRINS	PENTOSANS	HEMICELLULOSE
1936		°F.	%	%	%	%	%	%
Feb. 11	Ohio	34 (check)	2.40	3.13	5.84	1.78	7.61	8.50
	French	34 (check)	3.33	4.11	7.85	1.63	7.12	8.47
Feb. 22	Ohio	34	1.71	7.32	9.56	1.03	7.03	8.13
" "	French	34	1.81	4.65	6.87	0.50	8.60	8.75
" "	Ohio	31	2.41	8.39	11.40	1.43	7.36	8.40
" "	French	31	2.23	6.93	9.68	1.21	7.64	8.91
" "	Ohio	12	3.31	1.40	4.94	1.04	8.21	8.60
" "	French	12	3.30	1.11	4.64	0.80	9.68	9.79

centage of increase and decrease of the pentosans and hemicellulose follow the decrease and increase of the total solids. This experiment was of considerable value and interest in that it showed that the changes that occurred in the roots of the undisturbed clover plants, when placed in darkness at temperatures of 34°, 31°, and 12° F., were similar to those in macerated tissue (autolysis). Especially is this similarity noted in the starch and dextrins fraction. The reducing sugars of both varieties of clover increased with the lowering of the temperature, the Ohio variety always maintaining the lead. The non-reducing sugars increased with the lowering of the temperature from 34° to 31° F., and then decreased from 31° to 12° F. The rate of starch and dextrin hydrolysis was greater in both varieties of clover at 34° and 12° F. than at 31° F.

The results of the nitrogen analyses are shown in table VII to XII. During autolysis a portion of the total protein in the roots and crowns is hydrolyzed into amino acids, bases, etc. The portion of the protein removed during the extraction of the non-protein (designated extracted protein) decreased in both root and crown tissue during the entire period of autolysis. The greatest decrease was during the first 24 hours of autolysis. Conversely the non-protein nitrogen increased. This increase in amino nitrogen at successive autolysis periods was fairly regular. However, basic nitrogen accumulated only during the later autolysis periods. There was little increase in the amide and ammonia fraction at any time.

In the January autolysis experiment the increase in non-protein nitrogen in both the Ohio and French roots was somewhat greater than the decrease in extracted protein. However, proteolytic enzyme activity was greater in the Ohio roots than in the French roots, as measured by the percentage increases in non-protein nitrogen over the original or check analysis (table VIII). Heating the sample to 70° C. for 1 hour did not destroy the proteolytic enzymes, although accumulation of non-protein nitrogen was not so great as in the untreated samples. The enzymes in the French roots were injured more than those in the Ohio roots by the heat treatment.

In the clover crown tissue which contained more total and protein nitrogen than the roots, there was a greater decrease in the extracted protein than an increase in the non-protein nitrogen. In further contrast with the clover roots, proteolytic enzyme activity was greater in the French crowns than in the Ohio crowns.

In March, after growth had started, the clover roots and crowns were lower in total nitrogen than in January. Proteolytic activity came to an equilibrium after 48 hours of autolysis in roots of both varieties. As in January, greater increases in non-protein nitrogen were observed in the Ohio roots than in the French roots. This increase was much greater than the decrease in extracted protein. Evidently the reserve proteins more intimately associated with the cellular structure were undergoing proteolysis.

TABLE VI

DISTRIBUTION OF CARBOHYDRATES IN RED CLOVER ROOTS. RESULTS ARE EXPRESSED AS PERCENTAGE OF THE ORIGINAL, TAKEN AS 100 PER CENT.

SAMPLING DATE	VARIETY	STORAGE TEMPERATURE	REDUCING SUGARS	SUCROSE	TOTAL SUGARS	STARCH AND DEXTRINS	PENTOSANS	HEMICELLULOSE
1936		°*F.*	%	%	%	%	%	%
Feb. 22	Ohio	34	71.3	233.9	163.7	57.9	92.4	95.6
" "	French	34	54.4	113.1	87.5	30.7	120.8	103.3
" "	Ohio	31	100.4	268.1	195.2	80.3	96.7	98.8
" "	French	31	66.7	168.6	123.3	74.2	107.3	105.2
" "	Ohio	12	137.9	44.7	84.6	58.4	107.9	101.2
" "	French	12	99.1	27.0	59.1	49.1	136.0	115.6

TABLE VII

DISTRIBUTION OF NITROGEN IN RED CLOVER ROOTS AND CROWNS SAMPLED JANUARY 30, 1935. RESULTS ARE EXPRESSED AS PERCENTAGE OF THE FRESH WEIGHT

PERIOD OF AUTOLYSIS	TOTAL N	PROTEIN N	EXTRACTED PROTEIN N	NON-PROTEIN N	ALPHA-AMINO N	BASIC N	AMIDE N	RESIDUAL N
Hours	%	%	%	%	%	%	%	%
			OHIO ROOTS					
(Check) 0	0.630	0.337	0.147	0.293	0.125	0.079	0.056	0.112
24	.....	0.247	0.089	0.383	0.157	0.071	0.064	0.162
1 hr. 70° C.; 24 hr. 37° C.	.....	0.261	0.086	0.369	0.141	0.076	0.066	0.162
48	.....	0.251	0.061	0.379	0.165	0.099	0.066	0.148
120	.....	0.178	0.049	0.452	0.199	0.186	0.076	0.177
			FRENCH ROOTS					
(Check) 0	0.544	0.296	0.129	0.248	0.108	0.065	0.052	0.088
24	.....	0.236	0.068	0.308	0.134	0.063	0.056	0.118
1 hr. 70° C.; 24 hr. 37° C.	.....	0.274	0.059	0.270	0.112	0.052	0.053	0.105
48	.....	0.216	0.064	0.328	0.147	0.067	0.062	0.119
120	.....	0.207	0.045	0.337	0.152	0.107	0.062	0.123
			OHIO CROWNS					
(Check) 0	0.649	0.402	0.218	0.247	0.118	0.060	0.066	0.063
24	.....	0.363	0.079	0.286	0.135	0.049	0.066	0.085
48	.....	0.332	0.062	0.317	0.153	0.075	0.070	0.094
			FRENCH CROWNS					
(Check) 0	0.593	0.384	0.206	0.209	0.100	0.056	0.055	0.054
24	.....	0.323	0.098	0.270	0.119	0.050	0.058	0.093
	.....	0.305	0.079	0.288	0.133	0.063	0.063	0.092

TABLE VIII

DISTRIBUTION OF NITROGEN IN RED CLOVER ROOTS AND CROWNS SAMPLED JANUARY 30, 1935. RESULTS ARE EXPRESSED AS PERCENTAGE OF THE ORIGINAL, TAKEN AS 100 PER CENT.

PERIOD OF AUTOLYSIS	PROTEIN N	EXTRACTED PROTEIN N	NON-PROTEIN N	ALPHA-AMINO N	BASIC N	AMIDE N	RESIDUAL N
Hours	%	%	%	%	%	%	%
			OHIO ROOTS				
24	73.3	60.5	130.7	125.6	89.9	114.3	144.6
1 hr. 70° C.; 24 hr. 37° C.	77.4	58.5	125.9	112.8	96.2	117.9	144.6
48	74.5	41.5	129.4	132.0	125.3	117.9	132.1
120	52.8	33.3	154.3	159.2	235.4	135.7	158.0
			FRENCH ROOTS				
24	79.7	52.7	124.2	124.1	96.9	107.7	134.1
1 hr. 70° C.; 24 hr. 37° C.	92.6	45.7	108.9	103.7	80.0	101.9	119.3
48	73.0	49.6	132.3	136.1	103.1	119.2	135.2
120	69.9	34.9	135.9	140.7	164.6	119.2	139.8
			OHIO CROWNS				
24	90.3	36.2	115.8	114.4	81.7	100.0	134.9
48	82.6	28.4	128.3	129.7	125.0	106.1	149.2
			FRENCH CROWNS				
24	84.1	47.6	129.2	119.0	89.3	105.4	172.2
48	79.4	38.4	137.8	133.0	112.5	114.6	170.4

TABLE IX

DISTRIBUTION OF NITROGEN IN RED CLOVER ROOTS AND CROWNS SAMPLED MARCH 25, 1935. RESULTS ARE EXPRESSED AS PERCENTAGE OF THE FRESH WEIGHT

PERIOD OF AUTOLYSIS	TOTAL N	PROTEIN N	EXTRACTED PROTEIN N	NON-PROTEIN N	ALPHA-AMINO N	BASIC N	AMIDE N	RESIDUAL N
Hours	%	%	%	%	%	%	%	%
			OHIO ROOTS					
(Check) 0	0.452	0.257	0.091	0.195	0.082	0.083	0.033	0.080
24	.....	0.228	0.051	0.224	0.089	0.075	0.032	0.103
48	.....	0.174	0.042	0.278	0.115	0.103	0.041	0.122
72	.....	0.174	0.040	0.278	0.116	0.145	0.039	0.123
			FRENCH ROOTS					
(Check) 0	0.405	0.246	0.080	0.159	0.060	0.068	0.025	0.074
24	.....	0.187	0.049	0.218	0.084	0.085	0.031	0.103
48	.....	0.181	0.038	0.224	0.090	0.089	0.034	0.100
72	.....	0.184	0.037	0.221	0.089	0.129	0.031	0.101
			OHIO CROWNS					
(Check) 0	0.495	0.359	0.210	0.136	0.062	0.043	0.033	0.041
24	.....	0.284	0.103	0.211	0.091	0.057	0.038	0.082
48	.....	0.266	0.071	0.229	0.106	0.065	0.041	0.082
72	.....	0.255	0.056	0.240	0.114	0.097	0.042	0.084
			FRENCH CROWNS					
(Check) 0	0.525	0.411	0.237	0.114	0.049	0.036	0.023	0.042
24	.....	0.329	0.111	0.196	0.084	0.051	0.033	0.079
48	.....	0.312	0.086	0.213	0.098	0.069	0.035	0.080
72	.....	0.306	0.074	0.219	0.103	0.088	0.036	0.080

TABLE X

DISTRIBUTION OF NITROGEN IN RED CLOVER ROOTS AND CROWNS SAMPLED MARCH 25, 1935. RESULTS ARE EXPRESSED AS PERCENTAGE OF THE ORIGINAL, TAKEN AS 100 PER CENT.

PERIOD OF AUTOLYSIS	PROTEIN N	EXTRACTED PROTEIN N	NON-PROTEIN N	ALPHA-AMINO N	BASIC N	AMIDE N	RESIDUAL N
Hours	%	%	%	%	%	%	%
	OHIO ROOTS						
24	88.7	56.0	114.9	108.5	90.4	97.0	128.8
48	67.7	46.2	142.6	140.2	124.1	124.2	152.5
72	67.7	44.0	142.6	141.5	174.7	118.2	153.8
	FRENCH ROOTS						
24	76.0	61.2	137.1	140.0	125.0	124.0	122.6
48	73.6	47.5	140.9	150.0	130.9	136.0	119.0
72	74.8	46.2	139.0	148.3	189.7	124.0	120.2
	OHIO CROWNS						
24	79.1	49.0	155.2	146.8	132.6	115.2	200.0
48	74.1	33.8	168.4	171.0	151.2	124.2	200.0
72	71.0	26.7	176.5	183.9	225.6	127.3	204.9
	FRENCH CROWNS						
24	80.0	46.8	171.9	171.4	141.7	143.5	188.1
48	75.9	36.3	186.8	200.0	191.7	152.2	190.5
72	74.5	31.2	192.1	210.2	244.4	156.5	190.5

In the clover crown tissue, proteolysis continued up to 72 hours. As in January, greater percentage increases in non-protein nitrogen were observed in the French crowns than in the Ohio crowns. The decrease in extracted protein again was greater than the increase in non-protein nitrogen.

TABLE XI

NON-PROTEIN NITROGEN IN RED CLOVER ROOTS AND CROWNS EXPRESSED AS PERCENTAGE OF THE TOTAL NITROGEN

SAMPLED JANUARY 30, 1935		SAMPLED MARCH 25, 1935	
PERIOD OF AUTOLYSIS	NON-PROTEIN N	PERIOD OF AUTOLYSIS	NON-PROTEIN N
Hours	%	*Hours*	%
	OHIO ROOTS		OHIO ROOTS
(Check) 0	46.51	(Check) 0	43.14
24	60.79	24	49.56
1 hr. 70° C.; 24 hr. 37° C.	58.57	48	61.50
48	60.16	72	61.50
120	71.75	.....	.....
	FRENCH ROOTS		FRENCH ROOTS
(Check) 0	45.59	(Check) 0	39.26
24	56.62	24	53.83
1 hr. 70° C.; 24 hr. 37° C.	49.63	48	55.31
48	60.29	72	54.57
120	61.95	.....	.....
	OHIO CROWNS		OHIO CROWNS
(Check) 0	38.06	(Check) 0	27.47
24	44.07	24	42.63
48	48.84	48	46.26
.....	.....	72	48.48
	FRENCH CROWNS		FRENCH CROWNS
(Check) 0	35.24	(Check) 0	21.71
24	45.53	24	37.33
48	48.57	48	40.57
.....	.....	72	41.71

A comparison of relative enzymatic activity in January and in March is furnished by calculation of the non-protein nitrogen as percentages of the total nitrogen of the root and crown tissue. These data are shown in table XI. Autolysis of the roots for 120 hours in January resulted in hydrolysis of protein to the extent of more than 25 per cent. of the total nitrogen of the Ohio roots and but 16 per cent. of the total nitrogen of the French roots. In March the corresponding figures were 18 per cent. for the Ohio roots and 15 per cent. for the French roots during an autolysis period of 72 hours.

TABLE XII

DISTRIBUTION OF NITROGEN IN RED CLOVER ROOTS

SAMPLING DATE	VARIETY	STORAGE TEMP.	TOTAL N	PROTEIN N	EXTRACTED PROTEIN N	NON-PROTEIN N	ALPHA-AMINO N	AMIDE N	RESIDUAL N
1936		°F.	%	%	%	%	%	%	%
			PERCENTAGE OF FRESH WEIGHT						
Feb. 11	Ohio	34 (check)	0.456	0.260	0.126	0.196	0.096	0.045	0.055
" "	French	34 (check)	0.452	0.256	0.137	0.196	0.089	0.041	0.066
Feb. 22	Ohio	34	0.426	0.216	0.140	0.210	0.095	0.041	0.074
" "	French	34	0.377	0.228	0.125	0.149	0.067	0.033	0.049
" "	Ohio	31	0.470	0.254	0.121	0.216	0.097	0.043	0.076
" "	French	31	0.501	0.288	0.157	0.213	0.092	0.044	0.077
" "	Ohio	12	0.589	0.402	0.125	0.187	0.084	0.039	0.064
" "	French	12	0.518	0.389	0.100	0.129	0.055	0.025	0.049
			PERCENTAGE OF DRY WEIGHT						
Feb. 11	Ohio	34 (check)	2.840	1.618	0.786	1.222	0.598	0.281	0.343
" "	French	34 (check)	3.178	1.800	0.963	1.378	0.626	0.288	0.464
Feb. 22	Ohio	34	2.969	1.507	0.975	1.462	0.662	0.286	0.514
" "	French	34	2.890	1.747	0.959	1.143	0.514	0.253	0.376
" "	Ohio	31	2.950	1.593	0.760	1.357	0.609	0.270	0.478
" "	French	31	3.050	1.754	0.956	1.296	0.560	0.268	0.468
" "	Ohio	12	2.819	1.925	0.598	0.894	0.402	0.187	0.305
" "	French	12	2.822	2.120	0.544	0.702	0.299	0.136	0.267
			PERCENTAGE OF TOTAL NITROGEN						
Feb. 11	Ohio	34 (check)	.....	57.02	27.63	42.98	21.05	9.87	12.06
" "	French	34 (check)	.....	56.64	30.31	43.36	19.69	9.07	14.60
Feb. 22	Ohio	34	.....	50.70	32.86	49.30	22.30	9.62	17.38
" "	French	34	.....	60.48	33.16	39.52	17.77	8.75	13.00
" "	Ohio	31	.....	54.04	25.74	45.96	20.64	9.15	16.17
" "	French	31	.....	57.49	31.34	42.51	18.36	8.78	15.37
" "	Ohio	12	.....	68.25	21.22	31.75	14.26	6.62	10.87
" "	French	12	.....	75.10	19.31	24.90	10.62	4.83	9.45

In January slightly less than 11 per cent. of the total nitrogen in the Ohio crowns was hydrolyzed in 48 hours. For the same period more than 13 per cent. of the total nitrogen in the French crowns was hydrolyzed. An autolysis period of 48 hours in March brought about hydrolysis of nearly 19 per cent. of the total nitrogen in both Ohio and French crowns.

The distribution of nitrogen in red clover roots stored for 11 days in darkness at low temperatures is shown in table XII. In this experiment the plants were undisturbed until sampled for analysis. Regardless of storage temperature the Ohio roots maintained a larger proportion of their total nitrogen as non-protein nitrogen than did the French roots. In the autolysis studies the Ohio roots were always higher in non-protein nitrogen than were the French roots. Proteolysis occurred in the Ohio roots stored at 34° F. or 31° F. A storage temperature of 12° F. resulted in a marked decrease in the proportion of non-protein to total nitrogen as compared with the original or check analysis. Even greater decreases were noted in the French roots stored at 12° F. To a lesser extent the same decrease was observed in French roots stored at 31° or 34° F. It is not possible with the available data to explain these decreases in non-protein nitrogen.

Discussion

The hardier variety (Ohio) contains a larger percentage of sugars than the less hardy (French) under cold-hardened conditions. A further interesting question is whether this greater quantity of sugar is due primarily to greater conversion of starch and dextrins to sugar, or whether the hardier variety is instead more conservative of the sugar already formed. This investigation indicates that the hardier clover is more conservative of its already formed sugar, as can be noted from tables I to VI. NEWTON and ANDERSON (**6**) have established varietal differences in wheat plants by means of their metabolic rates at temperatures below 0° C. A similar relationship seems to exist between hardy and less hardy varieties of red clover.

It is very difficult to learn to what extent enzymatic hydrolysis of the carbohydrates in macerated red clover roots and crowns can be correlated with naturally occurring enzymatic activity in plants grown under controlled or field conditions. The data of tables V and VI show that the changes that occurred in the roots of the undisturbed clover plants when placed in darkness at temperatures of 34°, 31°, and 12° F. were similar to those in macerated tissue (autolysis). In a previous publication (**3**) it was reported that the Ohio variety of red clover grown under field conditions maintained a higher level of sugars and starch than the French variety. The present investigation shows that the enzymes active on starch in the French variety were capable of hydrolyzing more starch in a given length of time than were those in the Ohio variety. Thus, it would appear that the

carbohydrate enzymatic activity in macerated clover tissue was similar to that naturally occurring in plants under field conditions.

There is a difference in the protective action between the two varieties of red clover. The hardy variety showed a greater protective action than the less hardy variety, after heating the tissue for 1 hour at 70° C. Exposure of the tissue to −22° C. for 1 hour did not influence the enzymatic activity when compared with the check. A number of investigators have attempted to correlate total sugar, amino acids, inorganic salts, pH, etc., and protective action to heat and cold by the addition of these substances to plant saps. This investigation shows a greater protective action with a greater quantity of sugar and non-protein nitrogen. A critical study of the percentages of these substances in the tissue will reveal the fact that any one factor such as sugar, etc., is not responsible for the difference in protective action, of the tissue at 70° C. for 1 hour. Undoubtedly, the ability of the enzyme complex to withstand heat is a test directly or indirectly of the stability of the entire cell-constituent complex.

In a previous publication (**3**) it was reported that, with the approach of winter, French red clover roots increased in non-protein nitrogen more rapidly than did Ohio roots. Whether this increase in non-protein nitrogen occurred through proteolysis or simply by accumulation of soluble nitrogen not yet organized into protein cannot be determined. The present investigation shows that the proteolytic enzymes in the Ohio clover roots were capable of hydrolyzing more protein in a given length of time than were those in the French clover roots.

Similarly under field conditions proteolysis was more rapid in the Ohio crowns than in the French crowns (**3**). Yet in the autolysis experiment greater proteolysis occurred in the French crowns than in the Ohio crowns.

It is considered that a high level of sugars in plants helps to prevent protein precipitation. The writers have shown (**2, 3**) that the Ohio variety of red clover is nearly always higher than the French variety in sugars and starch. At the same time the French variety usually maintains the higher proportion of its total nitrogen in the form of protein.

It is quite possible that the analytical determinations designed as indices of cold-hardiness are not of equal value for all plant species.

Summary and conclusions

1. The results of this investigation suggest that the adaptability of the red clover plant to winter conditions is closely associated with its rate of carbohydrate metabolism. The enzymes active on starch in the French variety (less hardy) were capable of hydrolyzing a greater proportion of the starch in a given length of time than were those in the Ohio variety. The proteolytic enzymes in the Ohio clover roots were more active than were those

in the French clover roots. A reverse relationship existed for the proteolytic enzymes in the crown tissue.

2. Heating the macerated clover tissue to 70° C. for 1 hour did not destroy the carbohydrate and proteolytic enzymes, although accumulation of sugars and non-protein nitrogen was not so great as in the untreated samples. The enzymes in the French variety of red clover were injured more than those in the Ohio variety by the heat treatment. This protected enzymatic activity is correlated with larger percentages of sugar and amino nitrogen in the Ohio variety than in the French variety.

3. Exposure of the tissue to – 22° C. for 1 hour previous to autolysis did not greatly influence the enzymatic activity, when compared with the check.

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WATER ABSORPTION IN THE COTTON PLANT AS AFFECTED BY SOIL AND WATER TEMPERATURES[1]

C. H. ARNDT

Introduction

In experiments at the South Carolina Experiment Station with solution cultures of cotton (*Gossypium hirsutum* L.) it was occasionally noted in early spring that severe wilting occurred in the morning when the greenhouse temperature had fallen as low as 16° C. during the preceding night. This unusual wilting was apparently due to sunlight causing a rapid rise in air temperature when the solution temperature was low; for the plants recovered quickly when the air temperature was lowered by ventilation until the solutions had become warmed to about 20° C. Similar morning wilting was observed in plants growing in soil when soil temperature was much lower than a rapidly rising air temperature. Because the relation of root absorption to soil temperature is of great importance in the study of plant-water relations and also because the soil and solution temperatures which led to the wiltings just mentioned are higher than those reported for other plants, a study was undertaken to obtain information concerning the relation of root temperature to wilting in the cotton plant under otherwise favorable conditions for its healthy functioning. Some results of that study are briefly set forth in this paper, in the preparation of which the author has been aided by Professor BURTON E. LIVINGSTON of the Johns Hopkins University.

Materials and methods

Observations were made on plants growing in solution and soil cultures. In the latter, young, vigorous, six-week-old plants were used; these had been grown in sheet-metal cylinders (25 cm. in diameter and 30 cm. deep), containing 18 kg. of a fertile sandy-loam soil. The average water content of the soil was held at 60 per cent. of the water-holding capacity, the water loss being replaced semi-weekly. To cool the soil, the cylinders were set in a tank of colder water, the water surface being about 3 cm. above the level of the free soil surface. The water temperature was maintained at approximately 1° below the desired soil temperature by means of an electric automatic water heater and a manually controlled stream of cold water from a refrigerating machine. When the desired soil temperature had been reached, the water bath was raised to that temperature. When lowering of soil temperature was not over 10°, approximately uniform soil tempera-

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tures could be established throughout the soil mass in 5 hours. The soil temperature was measured at a depth of 4 cm. in the axis of the cylinder; if the temperature of parts of the root system differed from this, it was undoubtedly always slightly lower rather than higher, and the total difference was never more than one or two degrees.

In the solution-culture studies, 8-week-old plants, averaging 60 cm. in height, were used. These were well branched and leafy, with numerous floral buds and even young bolls. Two-liter Erlenmeyer flasks were used as containers, a single plant in each. The solution employed, which had been found quite satisfactory for the cotton plant, had the following composition: KNO_3, 0.0014*M*; $Ca(NO_3)_2$, 0.0028*M*; KH_2PO_4, 0.0007*M*; and $MgSO_4$, 0.001*M*; ferric citrate, 0.00001*M*; $MnSO_4$ and $Na_2B_4O_7$, each 0.00002*M*; and $CuSO_4$, 0.0000005*M*. The solutions were renewed semi-weekly, water being added as needed to maintain a solution volume of 2 liters. To prevent excessive heating of the solutions by sunlight, each flask was placed in a 9-liter stone-ware jar, with excelsior packing, both jar and flask being covered with a cone of thick paper with a central opening for the plant. During the growth period the solution temperature showed a daily range of approximately 20°–35°. The solution temperature was lowered by a procedure similar to that employed for the soil cultures. During the cooling, heat transfer was accelerated by slowly bubbling air through the solution and by an occasional shaking. In other experiments cooling was accelerated by replacing the solution with a fresh one at the desired temperature before placing the flask in the water bath.

When roots were to be heated, a large cylindrical water bath was brought to the desired temperature before the plant container was suspended in it. This temperature was maintained throughout the experiment by means of a manually controlled electric heater. The container fitted closely into the cover of the bath and a large circular sheet of cardboard was fitted closely around the base of the plant, to prevent heated air from rising around the plant.

Air temperature and humidity were recorded by means of a shaded hygrothermograph adjacent to the plants on which the observations were being made.

Experimentation

Unless otherwise noted, all experiments here described were performed in full sunlight in an unshaded greenhouse between April 1 and August 25, 1933. Wilting of the plants first became evident as a loss of turgidity of the leaves and a drooping of their margins. At a more advanced stage (recorded as severe wilting) there was noticeable drooping of the petioles and ends of the branches. During the heat-treatments the areas between the veins usually became noticeably bleached. These bleached areas disappeared on

cooling the roots, only to reappear several days later as yellow to brown areas. Such affected leaves usually dropped from the plant within a week.

Wilting induced by sudden lowering of solution temperature

In twelve tests in which the temperature of the root system was suddenly lowered by replacing the old solution with a new and colder one, the latter being from 10° to 23° colder than the former, wilting began within 20 to 75 minutes. The length of this period showed no consistent relation to (a) the solution temperature at which wilting occurred (which varied from 11° to 16°), to (b) the initial solution temperature (which varied from 28° to 36°), to (c) the temperature lowering to which the roots were subjected (which varied from 10° to 23°), to (d) the current air temperature (which varied from 32° to 36°), to (e) the difference between root and current air temperature (which varied from 17° to 24°), or to (f) the current relative humidity (which varied from 25 to 50 per cent.). The following examples illustrate the actual results of specific experiments:

(1) Solution temperature lowered from 28° to 14°; wilting observed in 20 min., at air temperature 38°, relative humidity 35 per cent.; solution was then allowed to become warmer and plant had fully recovered in 85 min., solution temperature then being 22°.

(2) Solution temperature lowered from 33° to 11°; wilting observed in 17 min., at air temperature 35°, relative humidity 45 per cent.; leaves flaccid and petioles drooped after additional 15 min.; plant fully recovered 3 hr. after removal from the cold bath when solution temperature had risen to 25°.

(3) Solution temperature lowered from 30° to 15°; wilting observed in 45 min., at air temperature 34°, relative humidity 50 per cent.; solution then allowed to become warmer, plant fully recovered in 45 min., when solution temperature had risen to 22.5°.

Wilting induced by gradual lowering of solution temperature

When the temperature of the roots was lowered gradually by immersing the flask in the cold bath without changing the solution, the processes leading to wilting went on concurrently with cooling, which was usually still in progress when wilting was first observed; at this time record was made of solution temperature and of the time elapsed since cooling began (which is here the same as the wilting time). In eleven tests in which initial solution temperature varied from 20° to 32° and time of cooling varied from 30 to 120 min. (with air temperature ranging from 30° to 39° and relative humidity from 20 to 50 per cent.), the plants wilted at various temperatures between 10° and 18.5°. There were no apparent relations among: (a) temperature difference between solution and air at time of wilting, (b) extent of cooling, and (c) wilting temperature; but larger solution-air differences generally

corresponded to more extensive cooling and to lower wilting temperatures. Conversely, none of the three variables just mentioned showed any consistent relation either to (d) wilting (cooling) time or to (e) rate of cooling, but the latter (d and e) were clearly related to each other; *i.e.*, when the rate of cooling was rapid the wilting time was short, and conversely, as might be expected. It may be said in general that, within the frame of these experiments, wilting began at solution temperatures between 10° and 18.5° and that it occurred more promptly when root temperature was lowered rapidly than when cooling was more gradual. The following detailed accounts are representative of the tests with gradually cooled solutions:

(1) Initial solution temperature 21°; wilting began after 30 min., at solution temperature 16.5°; air temperature 32° and relative humidity 35 per cent. at the beginning of visible wilting.

(2) Initial solution temperature 21°; solution cooled for 105 min., when wilting began, at solution temperature 15.5°; air temperature at beginning of visible wilting 35°, relative humidity 25 per cent.

(3) Initial solution temperature 30°; solution cooled for 30 min., when wilting began (11:15 A.M.), at solution temperature 18.5°; air temperature at beginning of wilting 38°, relative humidity 20 per cent. Solution was then allowed to warm to air temperature and recovery was complete before 2:45 P.M., when air temperature was 29.5°; solution was then cooled until it reached a temperature of 11.5°, after 110 min. (4:35 P.M.) but plant did not wilt; air temperature at this time 35°, relative humidity 40 per cent.; sky had been overcast since 2 P.M. Failure to wilt at this low solution temperature was apparently related to cloudiness (see below, effect of shading soil cultures at low soil temperature), decreased air temperature, and increased relative humidity.

(4) Initial solution temperature 25°; solution cooled for 40 min., when wilting began, at solution temperature 10°; air temperature at this time 35°, relative humidity 50 per cent. Solution was then held at 10° for 4 hours longer, at the end of which period (late afternoon) all leaves were flaccid and petioles and ends of branches drooped. Plant regained turgidity the following night with solution at the temperature of the greenhouse, but on the following morning some of the younger leaves were observed to be slightly blackened on their margins. Several days later areas between the veins of these same leaves and of some of the other young leaves, which had not yet reached their full size, became somewhat yellowish and remained so for several days. Subsequent growth was apparently normal, excepting that many young roots had been injured. These had lost their geotropic sensitiveness and elongated at right angles to the pull of gravity; they became somewhat hooked at the ends and each showed a marked constriction behind the tip. The injured roots elongated only slightly, but many healthy branches developed from their older portions.

WILTING INDUCED BY LOWERING OF SOIL TEMPERATURES

In studying the influence of unusually low soil temperatures on infection of cotton seedlings, wilting of healthy plants had been observed on clear days with high greenhouse temperature and low relative humidity at times when soil temperature was about 18° or lower. The soil temperature at which such wilting occurred was apparently always essentially the same whether the plants had been grown at constant soil temperatures (18°, 21°, 24°, 27°, 30°), or had been subjected to the daily temperature fluctuations of the greenhouse, or had been subjected to artificially controlled semi-daily temperature alternations (30° by day and 16° by night); also whether the holard at the time of wilting represented 80 per cent. of the water-holding capacity of the soil, or only 60 per cent. This wilting was observed, however, only in full sunlight; it was never observed on cloudy days, even when soil temperature was below 18° and air temperature was relatively high. Furthermore, plants that had wilted in this way on clear days regularly exhibited rapid recovery in the late afternoon when the intensity of solar radiation was rapidly diminishing.

Some special experiments were carried out concerning the effect of shading on the occurrence of wilting. Plants grown in the usual soil containers were arranged in pairs in the same cold water bath; one of each pair was left in full sunlight, while the other was shaded from direct sunlight by a 60-cm. square of thick cardboard supported horizontally 20 cm. above the plant, so that air circulation around the plant would not be greatly modified. These tests were made on clear days in July. The unshaded plants wilted more or less promptly at a soil temperature of 18°, but none of the shaded plants wilted. It therefore appears that intense sunshine and low soil temperature acted in conjunction to produce wilting. The following accounts are representative of these tests with shaded and unshaded plants.

(1) At 8:30 A.M. on July 23 a pair of similar plants were placed in the cold water bath (temperature 16°), where they remained until late in the afternoon of the next day. At 12:15 P.M. the unshaded plant was very noticeably wilted but the shaded one showed no wilting. Soil temperature was then 18° in the central axis, 16° at the margin, air temperature was 36° and relative humidity was 45 per cent. At 3:00 P.M. (air temperature 37°, relative humidity 35 per cent., soil temperature as above) the shaded plant was still unwilted but wilting of the unshaded plant was more pronounced than at 12:15 P.M. (At this time it was observed that similar cultures on a nearby greenhouse bench—unshaded and with soil temperature 41°—showed no wilting.) During the night the wilted plant recovered and appeared perfectly healthy at 8:30 A.M. the following morning, when soil temperature in the central axis was 17°, air temperature and relative humidity being 30° and 60 per cent. The leaves of the unshaded plant became gradually more

flaccid until 2:30 P.M., when the shade was removed. At that time (air temperature 36°, relative humidity 35 per cent.) the shaded plant remained apparently unaffected. At 4:45 P.M. both plants were severely wilted.

(2) July 7, 2:15 P.M., cultures with soil temperature 32° were placed in water baths at 18°, 20°, 22°, and 29°. At 5:00 P.M. (air temperature 38°, relative humidity 20 per cent.), when the soil masses had reached approximately the same temperatures as the baths, the plants in the 22° and the 29° baths were unwilted; those in the 20° bath (soil 22°) were slightly wilted; while those in the 18° bath (soil 20°) were badly wilted. At 8:30 A.M. the following day (air temperature 25°, relative humidity 55 per cent.), none of these plants was wilted, although the soil in the 18° bath had been cooled to 15° by cutting off the electric heater and increasing the supply of cold water. Because of inadequate cold-water supply, the temperature of that bath rose slowly and at 11:00 A.M. the soil had reached a temperature of 18° and the plants were slightly wilted (air temperature 33°, relative humidity 25 per cent.). At 3:00 P.M. these same plants were severely wilted (petioles drooping, youngest leaves more wilted than old ones), although the soil had reached a temperature of 20° at a depth of 4 cm. in central axis (air temperature 39°, relative humidity 15 per cent.). The plants in the 22° soil were unwilted. All wilted plants recovered quickly after sunset, even in the coldest bath, in which the soil temperature had again fallen to 18°.

(3) July 16, 10:00 A.M., six cultures were placed in water bath at 20°; at 2:00 P.M., with soil temperature approximately like that of bath, all plants were unwilted (air temperature 36°, relative humidity 20 per cent.). Three of these plants were then removed to a 17° bath. At 4:20 P.M. (air temperature 37°, relative humidity 20 per cent.) all plants in the 17° bath (soil 18°) were severely wilted, but those in the 20° bath (soil 20°) still showed no signs of wilting.

(4) July 15, 10:00 A.M., several cultures with soil temperature 22° were placed in bath at 16°. At 10:40 A.M. plants were severely wilted (soil temperature at 4 cm. depth 16° in center and 19° near margin; air temperature 32°, relative humidity 28 per cent.). Cultures were then removed to greenhouse bench and plants had completely recovered 35 min. later, at which time soil temperature near margin of cylinder had risen to 20° (air temperature and relative humidity approximately the same as at 10:40 A.M.).

In full sunlight, with low relative humidity and air temperature 30° to 40°, the plants generally wilted at soil temperatures from 16° to 20°, the higher soil temperatures being generally associated with the higher air temperatures and lower humidities. Thus the plants growing in soil wilted at slightly higher temperatures than those growing in solution cultures.

Wilting and other injuries induced by raising solution temperature to 60° C. or higher

Several earlier studies, which have been reviewed by Kramer (**12**), and to which he has added confirmatory observations on cotton and several other plants, have shown that plants, whose roots have been killed by high temperatures, may remain unwilted for several days. Some of the writer's results from experiments with heated roots are in essential agreement with the earlier observations, but some additional effects of the temporary application of high temperature to cotton roots are to be noted.

In routine solution cultures of cotton grown in the greenhouse, the solution temperature frequently reached 40° and remained so for several hours on bright sunny days with no apparent injury to either tops or roots. In special experiments, heating the solution for 75 min. at 50° produced no noticeable injury and no reduction in the rate of transpiration. Prompt wilting and subsequent additional symptoms of injury were produced, however, when the solution around the roots was heated to 70° during a 75-min. period and then held at that temperature for 15 min. For the first day after this treatment, a plant thus treated transpired at about the same rate as the unheated controls; but for the second day and the five succeeding days, its daily transpiration rates were respectively only 14, 31, 38, 19, 23, and 14 per cent. of the corresponding control rates. The effect of this heat treatment on transpiration was thus not immediate, but it became evident after 24 hours. The actual water loss after treatment, however, was considerable, being (for the 7 days in order) 115, 30, 60, 110, 50, 50, and 30 gm. On the last day of this record the transpiration for the control plant was 220 gm., or about seven times as great as that of the heated plant. Similar results were secured with respect to transpiration when the solutions were heated to 60°, except that the retardation of transpiration was more gradual and longer delayed; *e.g.*, a plant whose solution had been heated to 60° in 30 min. and kept at that temperature for 75 min. gave the following successive rates of water loss (expressed as percentages of the corresponding control rates): 95, 93, 37, 24, 19, 14 gm. Thus notable retardation was not noticeable until the third day, after which it became gradually more pronounced until both the 60°- and 70°-treated plants showed the same degree of retardation. These observations suggest that, although these heat treatments killed or greatly injured the roots, the latter were able to supply water to the rest of the plant at an adequate rate for a day or more after the treatment—the length of this period being shorter with the more severe treatment.

Additional symptoms of injury that accompanied or followed these heat treatments were readily observed. When the solutions were heated to 60° or higher, numerous gas bubbles escaped from the root lenticels while heating was in process, apparently because of gas expansion in intercellular

spaces. Also, wilting of the older leaves occurred while the 70° treatment was in progress, but wilting did not occur with the 60° treatments. The 70°-treated plants recovered from wilting during the night and were apparently healthy the following day. Concurrently, however, with the reduction of transpiration on the second day after treatment, wilting of the older leaves again became apparent, areas between the veins became discolored, and there was no subsequent recovery. All plants subjected to solution temperature of 60°, or higher, for a period of 60 min. showed conspicuous light-colored areas between the veins of the older leaves before the end of the heating period. After the solution or soil had cooled to air temperature these areas disappeared, but they reappeared a day or two later as conspicuous yellow or yellow-brown areas. Leaves so affected became abnormally rigid and leathery, never recovered, and eventually fell from the plant. The larger veins of the leaves of the 60°-treated plants retained their bright green color for several days after the discoloration of neighboring areas. Before a leaf dropped these veins often became darker brown than the rest of the leaf, appearing much like veins discolored through leaf infection by *Phytomonas malvacearum*. All such leaves were shed from the 70°-treated plant within a week; but they were retained somewhat longer (8–10 days) on the plants whose roots had been subjected to the 60° treatment. The youngest leaves showed no discolored areas and were retained longest. Some flowers opened quite normally, even after all but the very youngest leaves near the stem tip had fallen.

Roots that received the 70° treatment made no real growth subsequent to the treatment, although some of the tips enlarged noticeably, became bulbous, and retained their usual color for several days. All of them died and became brown within a week. Following the 60° treatment, some elongation of roots continued for several days; but the new root region formed was irregular in diameter and its apical portion tended to become bulbous, as when roots had been subjected to 10° for 4 hours. The older portions of the roots seemed to have been more severely affected by the treatment; they became dark brown and were apparently dead within a few days. After two weeks all roots were flaccid, gelatinous, and unquestionably dead.

Sections cut a week after the most severe treatment (70°) showed the xylem of the tap root to be dark brown, and brownish streaks were observed extending upward into the xylem of stem, branches, and petioles. Root cortex was obviously dead, but stem cortex was still green and showed no signs of injury. After a recovery period of three weeks, plants that had received the 60° treatment showed no discoloration of root xylem; but the root cortex was dead, excepting a small portion that had been above the level of the solution surface at the time of treatment. On the other hand, a brownish discoloration was evident in the xylem above the collet, in

streaks which were progressively less pronounced farther up. In a 60°-treated plant sectioned after 12 days, some discoloration was present even in the petioles. All leaves on the 60° plants at the time of treatment dropped before the end of three weeks. At that time there were some weak green shoots arising from the lower portion of the main stem.

Injury induced by raising soil temperature to 60° C. or higher

Experiments in which the soil temperature was temporarily raised to 60° or 63° gave results similar to those obtained with the heat-treated solutions. On clear warm days, when the aerial surroundings were such as to produce relatively rapid transpiration, these heat treatments of plants in soil always produced pale or nearly white areas between the larger veins of the leaves while the treatment was in progress. More or less wilting usually occurred during the treatment; in several instances even the tips of the branches lost their turgidity. When the soil was cooled to greenhouse temperature immediately after the treatment, the discolored areas between the veins disappeared and the plants regained their turgidity. No sign of wilting or discoloration was present on the day after treatment, but after an additional day or two those leaves that had become severely discolored during treatment became flaccid and did not regain turgidity. All regions that had become discolored during treatment and had then apparently recovered became pale yellow again at this time, drying up and turning brown within several days. All such discolored leaves dropped from the plant within two weeks. Similar discolored areas appeared on leaves that had not wilted during treatment but had shown discoloration at that time. As in the solution cultures, these leaves soon became unusually rigid and leathery and later dropped from the plant.

A typical example may represent these experiments with heated soil. At 2:00 P.M., a healthy plant (soil temperature 40°, holard 60 per cent.) was transferred from the greenhouse bench to a 60° water bath. At 6:00 P.M. (soil temperature at the 4 cm. depth 56° in center and 65° near margin) the older leaves were severely wilted and there was a pronounced discoloration between veins in many unwilted leaves as well as in the wilted ones. The plant was then returned to the greenhouse bench, where it regained turgidity and the discolored areas disappeared within several hours. There were no visible abnormalities the day after the treatment. Most of the discolored areas, however, again became very conspicuous on the second day, reappearing as yellowish areas, which became dry and brown within a few days. Leaves, thus affected, fell within 10 days; but the young leaves on the plant at the time of treatment were still apparently healthy at the end of 3 weeks, during which period new leaves had formed at the tip of the main stem. On removing the plant from the soil at this time it was noted that all of the roots

present at the time of treatment were dead, that the xylem throughout the root system was brown, and that this discoloration extended upward in the stem xylem for 12 cm. In spite of this severe injury, new roots were arising from the collet region immediately below the surface of the soil and new branches were forming on the main stem.

Discussion

References to the literature concerning the physiological effects of low soil temperatures may be found in the writings of KÖHNLEIN (**10**), WHITFIELD (**17**), PEIRCE (**16**), and CLEMENTS and MARTIN (**6**). It seems to be clear that wilting may be brought about, at least in a number of different plant forms, by a sufficient lowering of the root temperature in an adequately moist soil when transpiration is relatively rapid. CLEMENTS and MARTIN (**6**) induced visible wilting in sunflower at soil temperature of 4.5° (40° F.), but it required a still lower temperature to produce severe wilting. The only record of wilting at soil temperature above 4.5° appears to be that of KÖHNLEIN (**10**, p. 411), who noted partial wilting of sunflower at soil temperature of 17.5° or 18.5°, but the air temperature of his greenhouse was 42°; this is considerably higher than the air temperature prevailing when CLEMENTS and MARTIN noted wilting in the same plant and it is also considerably higher than the air temperature at which cotton plants wilted for the writer with soil temperature 20°. KÖHNLEIN also noted that his sunflower plants with soil at 18° wilted much more promptly than those with higher soil temperature in the same greenhouse.

It seemed remarkable that cotton plants wilted with root temperatures no lower than 18° under air conditions that were not unusually favorable for rapid transpiration, while they wilted at still higher root temperatures (20° to 21°) under air conditions that should have promoted unusually rapid transpiration. Different kinds of plants grown under similar conditions, and even plants of the same species grown under different environmental complexes, might be expected to wilt with different degrees of root cooling, even when all influential conditions except root temperature were quite healthful and alike at the time of wilting. It seems probable that, for any previously healthy individual plant, wilting induced by root cooling should occur with different degrees of cooling according to the combination of air temperature, air moisture, and radiation prevailing at the time of wilting. Such suppositions are based on the familiar consideration that wilting of this sort, which is immediately due to inadequacy of foliar moisture, is determined partly by the transpiration rate and partly by the rate at which water enters the leaves. In these root-cooling experiments the water-conducting capacity of the plants was surely not inadequate; neither was the environmental water supply inadequate, for the soil cultures were well supplied

with soil moisture and the water supply in the solution cultures was practically infinite. It therefore seems safe to suppose that these cold-root wiltings were occasioned by inadequacy in the capacity of the root systems to receive sufficient water from without and to permit its rapid movement into the conducting channels.

The capacity of a root system to receive water from the soil solution is of course dependent partly on the extent of the absorbing surfaces, partly on the rate of water movement from root to stem (essentially the current transpiration rate in such plants as cotton), and partly on the more or less obscure conditions that are effective within the root tissue between the absorbing periphery and the conducting channels. Lowering the root temperature, as in these experiments, surely could not have reduced the extent of the root surface, nor did it increase the transpiration rate. Consequently it appears that the cold treatment either reduced the capacity of the root tissue to receive water from an adequate external supply or else reduced its capacity to let water move into the vessels.

As to the manner in which the living cortex of the absorbing region of the root might possibly be markedly more resistant to water passage at 10° to 20° than at 30° to 40° a number of different hypotheses might be suggested, but to test them logically would require quantitative experimental data that are not yet available. Two possible hypotheses may be mentioned in a roughly qualitative way: (1) A very simple hypothesis may be based on the temperature relation of the viscosity of water, which is about one and one-half times as great at 20° as at 40°. At a specified pressure gradient water moves through finely porous material more rapidly as the temperature is raised. The pressure gradient by which water moved through the root cortex into the xylem in the experiments of this study could not have been increased, since negative pressure in plants becomes more pronounced with the approach of wilting. It follows that a reason for the slower water movement at lower than at higher root temperatures, as shown in these experiments, is to be sought in increased resistance rather than in decreased pressure gradient. According to this hypothesis such increased resistance is considered as due wholly to change in water viscosity; the colloidal structure and arrangements of non-water materials in the root being assumed as not effectively altered when root temperature is lowered. For some experimental findings concerning the influence of water viscosity on water movement through porous porcelain reference may be made to CHRISTIANSEN, VEIHMEYER and GIVAN (5). The observations of BODE (3) on the effect of temperature on the passage of water from soil through the roots of the sunflower to the stem and those of KRAMER (13) on the absorption of water from the soil by porcelain cones lend support to this hypothesis. (2) The temperature relations of solation and gelation in colloidal material might be

introduced as the basis for another qualitatively plausible hypothesis, if it is roughly supposed that the cortex cells interpose a greater resistance to water passage at lower than at higher temperature, not only because water is somewhat less fluid at lower temperatures but also because of changes in the ultramicroscopic arrangement, distribution, or configuration of their non-water material. Changes induced by lowering the temperature of the protoplasts may be readily supposed to render the entire tissue less permeable to water passage, as if the paths of water movement through them were narrowed or had become less numerous with decreasing temperature. Furthermore, it is at least logically possible that the ultramicroscopic porosity of the cell walls of the absorbing root cortex—including walls in immediate contact with the environmental water—may sometimes be reduced as the temperature is lowered.

It may be added that if the rate of water movement through the absorbing root cortex into the xylem (when the external water supply is adequate) is generally influenced by some sort of protoplasmic pumping action—as many writers have supposed—that action would be less effective as the water viscosity becomes greater and as permeability of the cortex and walls to water becomes less. Furthermore, the pumping action itself might be less vigorous at lower than at higher temperatures, which would be expected if such action really exists and if it is associated with respiratory activity—as suggested by LETA HENDERSON (**8**); for respiration is generally less rapid at lower than at higher temperature. But of course changes in cortex permeability—as those of solation and gelation—might be supposed to be greatly influenced by intensity of metabolic oxidation, whether pumping action is hypothesized or not. In this connection it is interesting to compare the soil temperature at which wilting occurred (20° to 21°) when conditions were favorable for rapid transpiration with the temperature relations of seed germination and subsequent growth in this tropical and subtropical species. CAMP and WALKER (**4**) and ARNDT (**1**) have observed that a soil temperature of about 27° was necessary for good top growth when air conditions were favorable, and FUNG (**7**) found that the optimal air temperature for top growth was 30° or higher. Some unpublished studies by the writer gave similar results. The writer has observed good root development at 24°, but then even at favorable air temperatures, top growth was retarded, as though the root systems were failing to supply the tops adequately with water or minerals, or both. Furthermore, germination percentage was found to be much reduced when the soil temperature was maintained at 18° to 19° (**4**, table IV; **1**), which is about the same as the minimal soil temperature at which the cotyledons could be raised above the soil surface. With a soil temperature between 18° and 20° the cotyledons that did appear above the soil remained yellow for a week or longer, as though the roots were not sup-

plying requisite materials at an adequate rate. It is thus seen that the critical soil temperature for wilting of cotton in the cold treatments described in this paper is not greatly different from what has been found to be the minimal temperature for healthy seed germination and seedling development, while it is only slightly below the soil temperature necessary for healthy root growth.

The results here presented on the effects of lethal high temperatures when applied to previously healthy plants are in essential agreement with previous observations, as reported for cotton and other plants by KRAMER (**12**), but the present account adds observations on previously unreported wilting of leaves at the time of the treatment, also on the bleaching of regions between the veins while heat treatment was in progress, and on the subsequent history of these injured regions. Wilting was observed while the heat treatment was being applied (a) when the solution was heated to 70° and maintained at that temperature for 15 minutes, also (b) when the soil temperature was raised to 56° or 65° (four hours being required to reach 56° in the center of the soil container after it had been placed in a 65° water bath). That this wilting was not due to heat effect on water viscosity is obvious. It is also obvious that whatever caused this loss of leaf turgor ceased to be operative shortly after the root temperature was reduced to 36°, for the treated plants soon recovered. It appears, however, that the rate of movement of water through the cortex was, in those cases, temporarily lowered in some way during heat treatment. This may suggest a reversible change of some kind in the root tissues, but since there is little information available in this connection it would be premature to attempt hypotheses concerning the possible nature of such a change.

That killing most of the root system by heat did not immediately reduce transpiration considerably (and subsequently reduced it only slowly) renders it probable that dead or severely heat-injured roots were still sufficiently absorptive and sufficiently permeable to water to keep the foliage adequately supplied for a considerable period. If some kind of pumping action dependent on healthy protoplasm in the roots were necessary for maintaining the transpiration stream at an adequate rate it might be expected that wilting should occur promptly with the application of heat to the root system, without the maintenance of the original transpiration rate for a day or more thereafter. For a somewhat thorough discussion of a similar problem to the one here suggested, reference may be made to OVERTON (**14, 15**) and KRAMER (**11, 12**). It is clearly apparent that the vessels of the heat-treated plants did not become seriously plugged—as by gum deposits—during the heat treatments nor for at least a day or two thereafter. They may have become plugged still later. BERKLEY and BERKLEY (**2**) observed that water still passed upward rapidly through the stems and petioles of

their cotton plants after these organs had been killed by high air temperatures. Some time after the roots were killed their previously living cells must have collapsed completely, but their coarser structure was apparently maintained as long as the original transpiration rate was not greatly reduced. Otherwise, it is difficult to imagine them maintaining adequate capillary contact with the moisture films of the soil.

Discoloration of the areas of the older leaves while the roots were receiving the injurious or lethal heat treatment may not have been due to an inadequate water supply of the leaves. When cotton plants wilted from the effects of drought, discoloration did not regularly occur and did not appear in plants wilted by application of low temperatures to the roots; but the discoloration here described was not unlike the permanent bleaching that may be produced by exposing cotton leaves to lethal air temperature, nor is it unlike the discoloration (called "scald" by the cotton farmer) that appears at times on leaves of cotton plants infected with *Fusarium vasinfectum*. The leaf discoloration that accompanied heat treatment of the roots was superficially suggestive of poisoning. Some sort of mildly toxic material formed in the heat-injured cells of the roots may perhaps have been promptly carried to the lower leaves in the transpiration stream—or possibly in the phloem. If such a supposition is tenable it must be supposed further that the first mildly injurious effect in restricted regions of the older leaves was not lethal and was reversible, for the discolored areas between the veins vanished within a few hours after the heat treatment was discontinued—within 90 min. in one experiment, in which the leaves had not wilted. Perhaps if the heat treatments had been more severe or longer continued such spots would not have regained their normal green color. At all events it seems quite possible that this early discoloration was due to some toxic material that arose in or near the heated roots and was carried upward quickly, that its delivery in the leaves ceased or became much slower very soon after heating was discontinued, and that its localized injurious effects in the foliar tissue were reversible in rather prompt recovery.

The second appearance of localized foliar discoloration, in the same areas as those previously discolored, also resembled some discolorations produced by poisons—for example, that produced by toxic concentrations of SO_2 on alfalfa in the experiments of HILL and THOMAS (9). This second discoloration sometimes first appeared as small, light brown spots (1 to 2 mm. in diameter), which rapidly coalesced to involve the entire area between adjacent large veins; the discoloration finally spread to the veins, as already noted. A similar discoloration of the leaves was noted on the cold-treated plants, but only when the temperature was lowered to 10°, which temperature was also sufficiently injurious to cause abnormal root development. In these latter plants, as contrasted to the heat-treated ones, the young leaves near the top

of the plant, which had not yet attained full development, were the first to show discoloration.

By the time of the second appearance of the brown areas on the leaves of plants whose roots had been heated, cell disintegration in the killed roots may have become greatly advanced and toxic material arising therein may have been of a more virulent type or more plentiful, or both, than during the short period of the heat treatment. The upward movement of such material might continue indefinitely, soon leading to local necroses in the affected leaves and to their eventual death. If these suppositions were approximately correct, it might be said, following OVERTON (**15**), of the affected regions and finally of these entire leaves, that they dried out because of death rather than that they died because of an inadequate water supply. It is, of course, not necessary to suppose the toxic material of the first evanescent discoloration during the period of heat treatment to have been at all of the same sort as that involved in the subsequent effects. It might be expected that the later injury should appear most promptly in tissues of localized regions that had been previously slightly affected and had apparently recovered; perhaps those tissues were originally unusually susceptible to injury of this sort and their recovery from the first poisoning may have been incomplete.

The brown stain, which soon appeared in the xylem of roots, stems, and even petioles of the heat-treated plants and its greater intensity and upward extent as the heat treatment was made more severe, may be taken as evidence that unusual material of some sort had passed upward from roots to foliage, for such stain has not been found in healthy plants. Discoloration immediately above the collet (*i.e.*, just above the most severely treated region) was usually darker than in the roots. It may be supposed that soluble substances not usually present in the transpiration stream were carried upward from the injured root systems, that some of these produced stain in the xylem vessels—perhaps also toxic effects in living cells of that tissue—and that some of them were responsible, first for localized injury in the leaves and then for the death of foliar tissue.

In connection with these hypothetical considerations concerning possible effects of toxic emanations from roots that had been killed or severely injured by heat treatment, some special attention should be given to the clearly differentiated but somewhat similar symptoms of leaf injury that followed excessive cooling of roots in these studies. Those symptoms were confined to the younger leaves, near the top of the plant, while it was the older leaves that were injured by excessive heating of roots. When the cold treatments were severe enough to cause abnormal root development (approximately 10°), the younger leaves were apt to show narrow regions of killed tissue at their margins on the day following the treatment and several days later

yellowish areas between the veins. There was no recovery of the marginal tissues, but the yellow areas between veins shortly disappeared, not leading to defoliation. The marginal injuries suggest effects of water deficiency and they might be regarded as analogous to tip burn. But the evanescent discoloration following cold treatment of the roots can hardly be attributed solely to excessive water deficit in localized regions, for such discoloration of young leaves is not commonly observed when plants are injured by ordinary drought when in the field. Perhaps these discolorations may indicate some form of mild poisoning, and it is at least logically possible to relate them tentatively to local accumulations—enhanced by foliar water deficit and transpiration—of toxic materials emanating from the injured roots. It will be recalled that these cold-treated plants exhibited some injury at the root tips and that the subsequent growth of many tips indicated physiological disturbance such as might result from disturbed metabolism and resultant toxic action.

Summary

1. Cotton plants growing in the greenhouse wilted on clear days when the temperature of the roots was sufficiently lowered for a short time. Solution cultures showed this wilting when their temperature had been lowered to between 10° and 18° C.; for soil cultures, with ample water supply, the highest soil temperature at which wilting occurred was generally somewhat higher, between 17° and 20°. The amount of this lowering necessary to produce wilting varied, being clearly dependent on air temperature, relative humidity, and sunlight intensity.

2. Exposure of the roots of plants growing in solution or soil cultures to a temperature of 60° for 60 min. severely injured the roots and caused a marked reduction in the absorption of water by such plants. Plants in solution cultures did not wilt while being heated to 60° in 60 min. A temperature of 70° maintained for 15 min., however, did cause similar plants to wilt. Plants growing in soil wilted at a soil temperature of 60°. White areas appeared between the veins of the leaves during the heat treatments at 60° and higher. These discolored areas disappeared within several hours after the reduction of the soil temperature, only to reappear several days later as yellow or brown regions in which the tissues became dry and died. Leaves thus affected dropped from the plants within 12 days.

3. The distal portions of some of the roots of plants in solution cultures were still capable of some limited elongation after an exposure to 60° for 60 min. The newly formed parts were, however, irregular in diameter and the roots became flaccid and gelatinous after several days. Exposure of roots to 10° for several hours caused them to lose their geotropic sensitivity, resulted in partial inhibition of growth in the apical portions, and produced

an abnormal bending and distortion of such new growth as did arise from the injured apices.

4. It is apparent that cooling of the roots produced wilting through lowering the capacity of those organs to absorb water from without and to transmit it to the conducting channels. Such lowering might be occasioned, to some extent at least, through increase in the viscosity of water, which accompanies cooling; also it might result from a decrease in permeability of root protoplasts or cell walls, due to lowered temperature; or it might result from retardation of some physiological activity of the root cortex (pumping action) induced by cooling. Wilting that occurred while the roots were at an injuriously high temperature may have been occasioned through lowered permeability of the root cortex or through production and upward movement of some sort of toxic material. Slight permanent leaf injury due to cold treatment of roots may perhaps have been due to inadequate water supply while the treatment was in progress, or possibly to toxic material. But the pronounced leaf injury and death of leaf tissues that followed several days after the heat treatment appears to have been due to injurious substances produced in the roots (most of which had been killed by the treatment) or possibly in the adjacent soil. This supposition is supported by the observation that death of heat-treated roots and post-mortem changes therein were accompanied and followed by pronounced discoloration of the stem xylem, which had not been heated.

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FACTORS IN VARIETAL SUSCEPTIBILITY OF WALNUT FRUITS TO ATTACK BY THE WALNUT-HUSK FLY

A. R. C. HAAS

(WITH SIX FIGURES)

Introduction

The varieties of Persian walnuts (*Juglans regia*) differ considerably in the susceptibility of their fruits to attack by the walnut-husk fly (*Rhagoletis completa*). BOYCE (1) has made an intensive investigation of the bionomics of this husk fly and has placed the varieties of walnuts in two groups according to the degree of their susceptibility:

VERY SUSCEPTIBLE	RESISTANT (SLIGHTLY SUSCEPTIBLE)
Eureka	Placentia
Franquette	Seedling
Klondike	Ehrhardt
Payne	Ware
	Neff

The maximum degree of hardness reached by husks during their development (as determined by the puncture-test method) was considered a varietal characteristic. The hardness of the husk was concluded to be the most important factor in determining the susceptibility of a variety to attack by the husk fly. The physical character of the husk in resistant varieties was found to be such that the fly could not puncture the surface layers in order to deposit eggs.

The present studies consider the nature of not only the physical but also the chemical factors involved in the varietal susceptibility of walnut fruits to attack by the husk fly. The results confirm the conclusions of BOYCE (1) regarding the importance of husk hardness and indicate the complexity of the factors involved in such susceptibility.

Materials

Walnut fruits of three varieties at various stages in their development were secured[1] from an orchard at Elsinore, California. In this location the fruits have not as yet encountered attacks of the husk fly. The varieties of walnut trees were interplanted and the cultural operations in the field were uniform. No dusts or sprays were applied to the fruits at any time.

[1] These fruits were secured through the kindness of Dr. R. S. REID, Oceanside, California.

Walnut fruits of nine varieties were collected on August 24, 1935, from trees growing near Chino,[2] California. These fruits were obtained from trees subject to attacks of the husk fly. Dusts or sprays had been applied to these trees during the season and possibly have had some effect on the husks.

Methods

Soon after the fruits were picked, they were rapidly washed in running distilled water and were wiped dry. The husks were at once removed[3] by means of stainless-steel knives. The removed husks were transferred to weighed Pyrex dishes (uncovered moisture chambers about 10 in. in diameter and 2 to 3 in. high). Drying was carried on at temperatures below 60° C. in a large well-ventilated oven. When dry the material was finely ground in a Wiley mill.

Husks of samples to be used for pH determinations were placed in rubber-stoppered dry bottles for two days in the freezing compartment of a refrigerator. The juice was then extracted from the husks at 25,000 lb. pressure and the pH values were determined by the quinhydrone electrode.

The number of fruits per sample, the fruit size, variety, fresh and dry weights of the husks, the shells, and the kernels were recorded. In the determination of the ash, the dry plant material was first charred in an electric muffle at low temperature and was then cooled. The salts soluble in hot water were then removed from the char and the residue heated until white. The solution was added to the cooled residues and was evaporated to dryness. After ignition the ash was cooled in a desiccator and then weighed.

Calcium was determined by titration of the oxalate with permanganate; magnesium was weighed as the pyrophosphate; reducing sugars were estimated by the SHAFFER-HARTMANN method, and total pectin was determined as calcium pectate by the method employed by BUSTON (**2**).

Experimentation

pH IN JUICE OF HUSKS

The pH was determined in the juice extracted from the husks of two collections of the Elsinore fruits. The results shown in table I indicate that the juices in the husks of fruits of a resistant variety such as Placentia are more acid (lower pH) than those in the husks of fruits of susceptible varieties such as Payne and Eureka.

[2] These fruits were collected by Dr. A. M. BOYCE, who was familiar with the varieties grown in that district. Grateful acknowledgment is made for this assistance.

[3] Even though rubber gloves were worn in removing the husks from the shells, the juice of the husks penetrated the gloves and stained the fingers so severely that painful flesh wounds were sustained.

TABLE I

DRY MATTER, ASH, AND pH OF WALNUT HUSK JUICE

DRY MATTER IN 100 CC. HUSK JUICE*		ASH IN 100 CC. HUSK JUICE*		pH OF HUSK JUICE*		pH OF HUSK JUICE†	
VARIETY	GRAMS	VARIETY	GRAMS	VARIETY	pH	VARIETY	pH
Neff	7.22	Neff	2.082	Franquette	6.82	July 19, 1935	
Franquette	6.76	Seedling	1.694	Payne	6.01	Placentia	4.70
Placentia	6.53	Payne	1.674	Klondike	5.72	Payne	4.96
Payne	6.33	Ware	1.468	Neff	5.66	Eureka	5.54
Ware	6.09	Klondike	1.447	Ehrhardt	5.65	August 20, 1935	
Seedling	6.07	Ehrhardt	1.359	Eureka	5.54	Placentia	5.20
Eureka	5.92	Franquette	1.276	Placentia	5.46	Payne	5.61
Ehrhardt	5.83	Placentia	1.179	Ware	5.38	Eureka	5.60
Klondike	5.13	Eureka	0.812	Seedling	5.30		

* Fruits collected August 24, 1935, at Chino, California.

† Fruits collected near Elsinore, California.

The results (table I) for the pH of the juices in the husks of nine varieties of walnut fruits obtained near Chino, indicate that, with one exception (Eureka), the varieties may be placed in the groups proposed by BOYCE (**1**, p. 379) for the susceptibility and resistance of the varieties to husk fly attack in the field. Increased acidity as found in the present studies in therefore associated with greater resistance.

TABLE II

DRY MATTER IN HUSKS AND SHELLS (WITH KERNELS) IN FRUITS COLLECTED AUGUST 24, 1935, NEAR CHINO, CALIFORNIA

HUSKS		SHELLS WITH KERNELS	
VARIETY	DRY MATTER IN FRESH WEIGHT	VARIETY	DRY MATTER IN FRESH WEIGHT
	%		%
Ehrhardt	9.03	Klondike	28.60
Seedling	10.16	Franquette	30.67
Klondike	10.21	Eureka	33.71
Ware	10.22	Ehrhardt	36.34
Neff	11.12	Payne	42.26
Placentia	11.14	Neff	43.47
Payne	11.33	Seedling	45.57
Eureka	12.25	Ware	46.14
Franquette	12.29	Placentia	46.65

TABLE III

DRY MATTER IN HUSKS AND SHELLS (WITH KERNELS) IN FRUITS COLLECTED DURING 1935 NEAR ELSINORE, CALIFORNIA

VARIETY	DRY MATTER IN THE FRESH WEIGHT OF HUSKS					DRY MATTER IN THE FRESH WEIGHT OF SHELLS WITH KERNELS				
	MAY 29	JUNE 7	JUNE 18	JULY 19	AUG. 20	MAY 29	JUNE 7	JUNE 18	JULY 19	AUG. 20
	%	%	%	%	%	%	%	%	%	%
Placentia	13.16	.....	14.11	12.92	10.83	8.25	.....	10.84	21.27	39.33
Eureka	.....	.....	14.71	14.74	11.36	.....	.....	9.69	24.72	24.50
Payne	.....	13.82	14.43	13.97	10.00	.....	9.53	11.61	20.79	38.65

Dry matter

In table II are given the percentages of dry matter in the fresh weight of the husks of fruits collected near Chino. With the exception of the Klondike, the varieties may be grouped according to the resistance grouping of Boyce (**1**, p. 379). The fresh husks of the more resistant varieties have the lower percentages of dry matter and conversely the higher percentages of water. Although the differences are small they are confirmed by the data given in table III for the Elsinore samples. The results (table III) indicate that the fresh husks of the susceptible varieties (Eureka and Payne) generally contain slightly greater percentages of dry matter than those of the resistant variety (Placentia).

When the shells and the contained kernels are considered (table II), the resistance is greater (Ehrhardt variety excepted) with the higher percentages of dry matter in the fresh weight. In the Elsinore samples this was the case only in the August collection.

It is of interest in table III that the percentages of dry matter in the fresh husks reach a maximum in late June and then decrease, while the percentages for the dry matter in the fresh shells (with kernels) increase throughout the season.

Table IV gives data regarding the weights of husks, shells, and kernels on which the computations of the various chemical analyses were based. In the Elsinore samples the dry weights per husk decreased after July 19 although in fruits of the Placentia and Payne varieties the fresh weights were still increasing. Also the dry weights of the shells (with kernels) and of the kernels increased at each fruit sampling although the fresh weights of the shells (with kernels) in Placentia and Payne decreased after July 19. It is of interest that the fresh weights of the husks and of the shells (with kernels) approximate one another at most times during the season and that after June 18 the dry weights of the shells (with kernels) greatly increase over those of the husks. The greater part of this increase occurs in the kernels.

The fresh weights of the husks of the fruits in the Chino samples (table IV) exceeded those of the shells (with kernels) in all varieties with the exception of the Klondike; the dry weights of the husks, however, were considerably less than those of the shells (with kernels).

Table I gives the grams of dry matter in the juice of husks in fruits of nine varieties. The juice was obtained as previously described and was filtered. No relation with the resistance of the husk to the attack of the husk fly was evident.

Ash, calcium, and magnesium

In table I are given the grams of ash in the filtered husk juice in fruits of nine varieties. No relation to husk fly resistance was found. It is seen

TABLE IV

RELATION OF HUSK TO SHELL AND KERNEL AT VARIOUS STAGES OF DEVELOPMENT

VARIETY	FRUIT SIZE	DATE OF COLLECTION	NO. OF FRUITS USED	AVERAGE WEIGHT PER HUSK		AVERAGE WEIGHT PER SHELL AND KERNEL		AVERAGE WEIGHT PER KERNEL
				FRESH	DRY	FRESH	DRY	DRY
	mm.	*1935*		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Placentia*	34 × 35 × 40	May 29	54	11.32	1.49	9.57	0.79	.....
	39 × 36.5 × 44	June 18	22	17.68	2.50	18.91	2.05	.....
	42 × 39 × 46.5	July 19	23	21.24	2.74	24.50	5.21	0.77
	42 × 39.5 × 46.5	August 20	22	21.44	2.32	23.29	9.16	3.94
Eureka*	35 × 33.5 × 46	June 18	47	14.43	2.12	13.32	1.29	.....
	41.5 × 40 × 52.5	July 19	25	28.00	4.13	22.45	5.55	0.80
	40 × 38 × 50	August 20	22	23.53	2.67	22.65	9.45	2.73
Payne*	32 × 34 × 40	June 7	44	14.85	2.05	10.07	0.96	.....
	40 × 37 × 50	June 18	32	18.45	2.66	23.86	2.77	0.18
	41 × 38.5 × 45.5	July 19	23	19.91	2.78	19.72	4.10	0.48
	41 × 39.5 × 46	August 20	22	23.18	2.32	19.56	7.56	2.33
Eureka		August 24	30	18.37	2.25	17.77	5.99	.....
Ehrhardt		August 24	25	21.18	1.91	17.06	6.20	2.33
Seedling		August 24	25	21.66	2.20	16.14	7.36	3.16
Franquette		August 24	25	21.90	2.69	17.63	5.41	1.27
Placentia		August 24	25	22.30	2.48	17.68	8.25	3.38
Payne		August 24	28	22.38	2.54	19.16	8.10	2.97
Ware		August 24	25	24.78	2.53	22.21	10.25	3.91
Klondike		August 24	26	25.50	2.60	33.42	9.67	2.28
Neff		August 24	27	27.57	3.07	24.83	10.79	3.71

* Fruits collected at Elsinore; the remainder collected at Chino.

from the percentages of ash in the dry matter of the husks (table V) that with the exception of the Placentia variety the high percentages are associated with high resistance to attack by the husk fly. These results are confirmed by the data given in table VI for the fruits collected at various times. With the exception of August 20, on which date the percentage for the Placentia variety is lower than that for the Payne (in agreement with the data in table V), the high percentages of ash are associated with increased resistance.

No association of resistance was found with the percentages of calcium in the dry matter of the husks (tables V, VI).

Except for the Payne variety in the August samplings, the high percentages of magnesium (tables V, VI) were related to increased resistance to husk fly attack. Table VI indicates that, prior to August, the percentages for the Payne and Eureka varieties are less than those for the Placentia.

The changes in the resistance position of the Placentia variety as regards ash and of the Payne as regards magnesium (table VI) is in confirmation of the results of BOYCE, who found that during the latter part of the season the hardness of the husks, which is a factor in resistance, may undergo considerable change.

TABLE V

ASH, CALCIUM, MAGNESIUM, POTASSIUM, SODIUM AND TOTAL PHOSPHORUS AS PERCENTAGES IN DRY MATTER OF HUSKS IN FRUITS COLLECTED AUGUST 24, 1935, AT CHINO, CALIFORNIA

VARIETY	ASH	VARIETY	CALCIUM	VARIETY	MAGNESIUM
	%		%		%
Seedling	15.40	Payne	0.82	Payne	0.226
Neff	14.06	Seedling	0.56	Ware	0.175
Ware	13.39	Placentia	0.54	Neff	0.125
Ehrhardt	13.32	Ehrhardt	0.53	Placentia	0.123
Klondike	13.20	Ware	0.52	Ehrhardt	0.110
Payne	12.00	Eureka	0.49	Seedling	0.093
Placentia	10.63	Neff	0.43	Franquette	0.087
Franquette	9.66	Franquette	0.33	Eureka	0.073
Eureka	8.28	Klondike	0.24	Klondike	0.064
VARIETY	POTASSIUM	VARIETY	SODIUM	VARIETY	TOTAL PHOSPHORUS
	%		%		%
Seedling	7.71	Seedling	0.27	Klondike	0.240
Klondike	7.04	Ehrhardt	0.24	Franquette	0.225
Neff	6.98	Neff	0.17	Neff	0.220
Ware	6.48	Ware	0.14	Ware	0.210
Payne	5.64	Klondike	0.12	Seedling	0.193
Placentia	5.30	Payne	0.10	Ehrhardt	0.138
Ehrhardt	5.23	Eureka	0.10	Eureka	0.130
Franquette	4.97	Placentia	0.09	Payne	0.115
Eureka	3.91	Franquette	0.03	Placentia	0.083

The percentages of ash in the husks of the samples from Elsinore (table VI) at first decrease and then increase. The greatest percentage gain in the ash occurred between July 19 and August 20. It is to be noted that, although the ash in the dry matter of the husks (table VI) reaches 12 to 14 per cent., the combined percentages for calcium and magnesium do not reach 1 per cent. HAAS (3) has shown that the dry matter of the husks of Placentia may contain over 10 per cent. of potassium (K), while the dry matter of leaves of this variety may contain less than 1.50 per cent.

POTASSIUM, SODIUM, AND TOTAL PHOSPHORUS

Table V shows the high percentages of potassium (K) found in the dry matter of the husks in fruits collected at Chino. With the exception of the Klondike, the varieties that show the greatest resistance to the husk fly attack generally contain the highest percentages of potassium. The Eureka and Franquette varieties are very susceptible and contain the lowest percentages of potassium. The Payne is only slightly out of the order shown in the field, in which this variety would occur between the Ehrhardt and Franquette. The percentages for the Payne, Placentia, and Ehrhardt varieties differ but little from one another.

The percentages for potassium (table VI) show the greatest increase

TABLE VI

ASH, CALCIUM, MAGNESIUM, POTASSIUM, SODIUM, AND TOTAL PHOSPHORUS AS PERCENTAGES IN DRY MATTER OF HUSKS IN FRUITS COLLECTED AT VARIOUS TIMES DURING 1935 AT ELSINORE, CALIFORNIA

IN DRY MATTER	VARIETY	MAY 29	JUNE 7	JUNE 18	JULY 19	AUG. 20
		%	%	%	%	%
Ash	Placentia	6.46	.....	5.27	7.12	13.09
	Eureka	.....	.....	4.97	6.58	12.40
	Payne	.....	5.77	4.76	7.05	13.60
Calcium	Placentia	1.02	.....	0.60	0.71	0.54
	Eureka	.....	.....	0.79	0.64	0.44
	Payne	.....	0.64	0.58	0.44	0.57
Magnesium	Placentia	0.214	.....	0.135	0.127	0.056
	Eureka	.....	.....	0.114	0.123	0.057
	Payne	.....	0.154	0.114	0.119	0.100
Potassium	Placentia	2.01	.....	2.16	3.06	6.80
	Eureka	.....	.....	1.76	2.79	6.41
	Payne	.....	2.32	1.87	3.30	6.71
Sodium	Placentia	0.06	.....	0.09	0.11	0.12
	Eureka	.....	.....	0.07	0.12	0.08
	Payne	.....	0.14	0.12	0.04	0.14
Total phosphorus	Placentia	0.175	.....	0.145	0.150	0.200
	Eureka	.....	.....	0.133	0.175	0.173
	Payne	.....	0.108	0.117	0.147	0.165

during the period from July 19 to August 20. As the results suggest, potassium is the chief basic constituent in the ash of walnut husks.

From the results (table VI) for the Elsinore samples, it is seen that on August 20 the husks of the Placentia and Payne varieties contain greater percentages of potassium in the dry matter than do those of the Eureka, which fact is in agreement with the results shown in table V.

In contrast with the high values found for potassium are the extremely low values found for sodium. In table V, with the exception of the Placentia variety, the more resistant varieties are associated with the higher percentages of sodium.

No relation was evident between resistance and the percentages of total phosphorus (tables V, VI).

TABLE VII

REDUCING SUGARS AND TOTAL PECTIN (AS CALCIUM PECTATE) IN WALNUT HUSKS OF CHINO FRUIT SAMPLES

VARIETY	REDUCING SUGARS IN DRY MATTER OF HUSKS	VARIETY	CALCIUM PECTATE IN DRY MATTER OF HUSKS
	%		%
Neff	9.62	Klondike	27.31
Payne	8.42	Neff	20.80
Placentia	7.74	Seedling	18.11
Ware	7.16	Placentia	17.79
Seedling	6.86	Ware	15.29
Franquette	6.46	Ehrhardt	13.64
Eureka	6.21	Franquette	11.89
Ehrhardt	5.66	Eureka	11.01
Klondike	2.70	Payne	7.33

REDUCING SUGARS AND TOTAL PECTIN

The percentages of reducing sugars in the dry matter of the husks of the Chino samples are given in table VII. The Payne and Ehrhardt varieties do not occur in the proper groups according to the resistance of these varieties in the field. Otherwise the high percentages of reducing sugars are associated with high resistance to attack by the husk fly.

In the fruit samples from near Elsinore (table VIII), the dry matter in the husks of the Placentia variety (resistant) in the July and August samples contained higher percentages of reducing sugars than the Eureka and Payne varieties (susceptible). The percentages in the August samples were less than those in the July samples.

In general the higher percentages of reducing sugars are associated with increased resistance. Analyses were also made of the total (as reducing) sugars in order to determine sucrose. The amounts present, however, were too small to permit of an accurate estimate.

TABLE VIII

REDUCING SUGARS AND TOTAL PECTIN (AS CALCIUM PECTATE) IN WALNUT HUSKS OF ELSINORE FRUIT SAMPLES

VARIETY	REDUCING SUGARS IN DRY MATTER OF HUSKS					CALCIUM PECTATE IN DRY MATTER OF HUSKS				
	MAY 29	JUNE 7	JUNE 18	JULY 19	AUG. 20	MAY 29	JUNE 7	JUNE 18	JULY 19	AUG. 20
	%	%	%	%	%	%	%	%	%	%
Placentia	7.15	.....	9.49	12.94	7.94	16.04	.....	12.45	13.83	16.09
Eureka	.....	.....	11.12	9.74	6.11	.....	.....	15.01	11.62	12.18
Payne	.....	10.58	9.76	10.82	5.87	.....	17.19	9.56	12.46	15.91

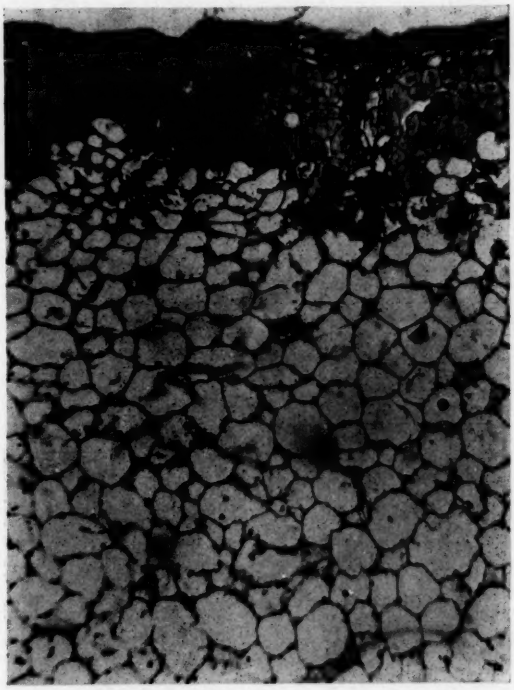

FIG. 1. Transverse section of husk of the Placentia variety (×144); wide, compact layer of thick-walled cells close to the epidermis.

The results for total pectin (as calcium pectate) in the dry matter of the husks in the Chino samples are given in table VII. With the exception of the Klondike variety, the high percentages are associated with increased resistance. The data (table VIII) for the July and August samples collected near Elsinore confirm this conclusion.

Pectin is found in the cell walls in leaves (**4**) and it is assumed to be a cell wall constituent in husks. The greater resistance to attack by the husk fly is associated with high percentages of pectin, and hence the cell wall structure may be a factor in determining husk resistance.

CELL STRUCTURE AND ARRANGEMENT OF TISSUES

In addition to the chemical analyses it seemed desirable to make determinations of the physical arrangement of certain tissues that might affect success in the puncturing of the husk by the fly.

The shells of the Payne variety of the Elsinore samples were becoming

hard on June 18, but still could be cut; the shells of the Eureka variety were very soft, while those of the Placentia were hard. The husks in some cases on August 20 could be slipped from the shell.

Sections were prepared of portions of the husks of the Elsinore fruit samples collected on August 20. Figures 1–6 are photographs of transverse sections of these husks. The magnifications for each variety are the same (×144 and ×593).

In the husks of the Placentia variety (fig. 1) the tissue that is composed of thick-walled cells is of greater width and compactness than in the husks of the Payne and Eureka varieties (figs. 3, 5). In the husks of the Eureka variety, stone cells are seen at intervals and oriented in a direction perpendicular to the surface.

The sections (×593) show conspicuous thick cell walls in the husks of the Placentia as compared with those in the husks of the other two varieties.

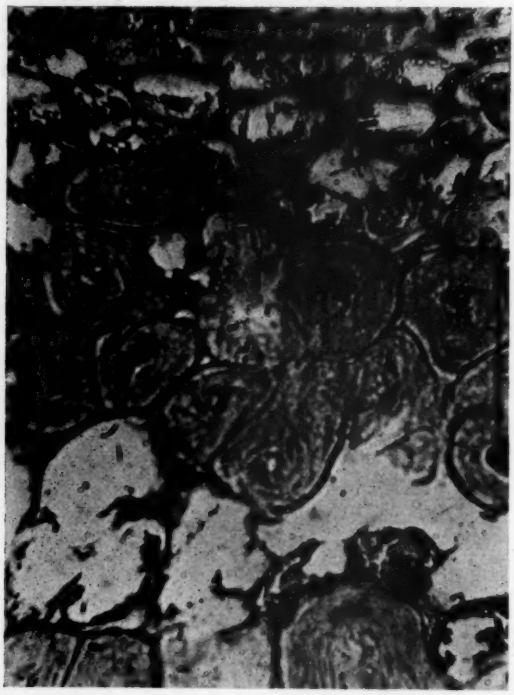

FIG. 2. Detailed section of Placentia husk tissue extending from epidermis to the heavy-walled cells (×593). The husk fly encounters difficulty in penetrating this thick-walled cell tissue.

FIG. 3. Transverse section of husk of the Payne variety (×144); relatively few layers of thick-walled cells.

The results for the cell structure and arrangement of tissues support the conclusion that the husks of fruits of the Placentia variety (resistant) are considerably more difficult for the walnut-husk fly to puncture than are those of fruits of the Eureka and Payne (susceptible).

Summary

1. A study was made of the complex of factors involved in the varietal susceptibility of walnut husks to attack by the walnut-husk fly. The importance of husk hardness as a factor (BOYCE **1**) is confirmed, and it is shown that there is a relation between this and other physico-chemical properties of husks in the different varieties.

2. Fruit collections were made at various times from three interplanted varieties of walnut trees located near Elsinore, California. These trees were not dusted or sprayed or subject to husk fly attacks. A single collection of fruits was made on August 24, 1935, from trees of nine varieties

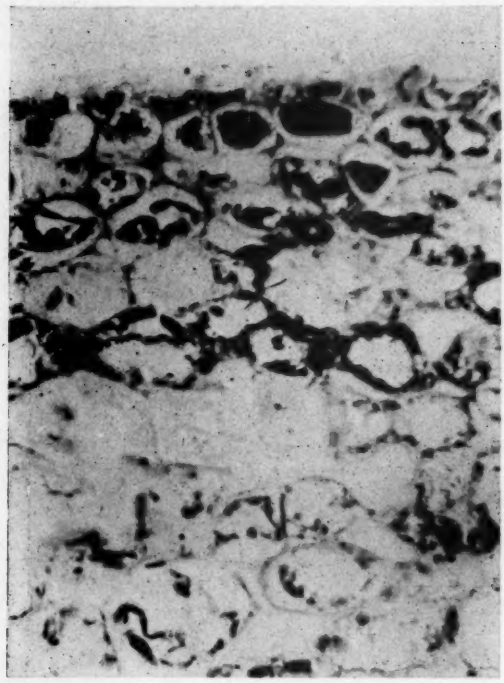

FIG. 4. Detailed section of Payne husk tissue extending from the epidermis (× 593), showing few, if any, thick-walled cells.

grown near Chino, California. These trees were subjected to dusts or sprays and to attacks of the husk fly.

3. With one exception (Eureka variety among the Chino samples) the pH of the juices of the husk appears to be related to the resistance to husk fly attack in the field. Increased acidity is associated with greater resistance.

4. Increased resistance is generally accompanied by a smaller percentage of dry matter in the fresh husks. In the August samples the resistance usually was associated with high percentage of dry matter in the fresh weight of the shells (with kernels).

5. The dry weights of the husks decreased after July 19 even though in fruits of the Placentia and Payne varieties the fresh weights were still increasing. The dry weights of the shells (with kernels) and of the kernels increased at each fruit sampling. The fresh weights of some varieties of shells (with kernels) decreased after July 19. The fresh weights of husks generally exceeded, while the dry weights were much less than the corresponding weights of the shells (with kernels).

FIG. 5. Transverse section of husk of the Eureka variety (×144); scattered, small thick-walled cells interspersed with clusters of heavy-walled cells oriented perpendicular to the epidermis.

6. No relation with the resistance to the attack of the husk fly was found in the grams of dry matter or in the grams of ask in the husk juices.

7. High percentages of ash, magnesium, potassium, sodium, reducing sugars, and total pectin in the dry matter of the husks were associated with increased resistance, while no relation of resistance to calcium or total phosphorus in the dry matter was evident.

8. Studies of transverse sections of the husks of Placentia, Eureka, and Payne varieties indicate the importance of cell-wall thickness and the distribution of these tissues. Husk hardness associated with resistance is largely due to the cell-wall structure and to the distribution of the husk tissues. Husks of fruits of the Placentia variety (resistant) possess thick-walled cell tissues of greater width and compactness than those of fruits of the Payne and Eureka varieties (susceptible).

9. Very little data exists in regard to the composition of walnut kernels,

FIG. 6. Detailed section of Eureka husk tissue showing very few thick-walled cells (×593).

shells, and husks. The present studies afford extensive data in regard to the composition of walnut husks.

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POLAR TRANSPORT OF AUXIN AND ELECTRICAL POLARITY IN COLEOPTILE OF *AVENA*

W. G. Clark

(WITH ONE FIGURE)

Introduction

In the introduction to a recent paper by the writer (**5**), the phenomenon of the polar transport of auxin in plants was briefly discussed. It was indicated there that the electrical theory of the polar transport of auxin was one of the most widely accepted ones. This theory states that the inherent electrical polarity of plants is the cause of the longitudinal polar transport of the negative ion of auxin, the plant-growth hormone, to an inherent positive pole in the plant.

It was concluded (**5**) that an electrical polarity exists in seedlings. Apices are electronegative to basal regions in seedlings of *Impatiens, Avena, Zea, Pisum,* and *Vicia.* In *Avena,* this polarity exists internally as well as on the surface of the cuticle. It is thus seen that the electrical polarity found in seedlings conforms with that demanded by the electrical theory of the polar transport of auxin.

Cut sections of these seedlings exhibited the same electrical polarity as intact plants, *i.e.,* electronegativity of the morphological apical cut surface. But it was also shown that this section-polarity could be inverted by gravity; and Clark (**4**) showed that the electrical polarity of the Avena coleoptile could be altered or inverted by light. Thus the electrical theory of polar transport is directly contradicted by the fact that neither light nor gravity affect the longitudinal transport of auxin (See "Discussion" and **5**).

The present article is concerned with experiments in which attempts were made to link polar transport and electrical polarity. Auxin transport and electrical polarity were measured at the same time, and then the electrical polarity was altered by applied potentials and by gravity to see if there were any parallel changes in polar transport.

Experimentation

Effect of applied P.D.'s on auxin diffusion in agar

In accordance with an electrical theory of transport of the plant-growth hormones, it was of primary importance first to demonstrate whether or not the hormone is electrically transported *in vitro.*

Dolk (**6**) attempted to demonstrate such an *in vitro* transport of auxin by the following method: *Zea Mays* growth substance (auxin-A) was collected by diffusion from coleoptile tips into agar blocks. Two such blocks

were interposed between platinum electrodes, and a P.D. of from 2 to 10 volts (v.) applied across them of one hour. At 10 v., a strong electroosmosis of water occurred, causing one block to take up water and swell, and causing the other block to lose water and shrink. DOLK stated that such electroosmosis would distort the results (the water current would carry the auxin to the negative pole). He also said that electrolytic destruction of auxin would probably occur at the electrodes. At 2 v., no appreciable electroosmosis occurred, but the auxin concentration in the two blocks remained the same, as tested by the standard *Avena* test (see text below). At 10 v., no auxin could be found owing to its destruction. DOLK concluded that auxin was not electrically transported under the conditions of his experiments.

KOCH (**12**) also investigated this problem. He placed 15 to 18 *Avena* coleoptile tips on agar blocks for 90 to 120 minutes to collect their auxin by diffusion, while passing a current from a 4-v. flashlight battery for 1 to 2 hours through the agar block between two electrodes (platinum wire). The block was then removed, cut into small blocks, and tested on *Avena,* using 2 plants per test. He claimed that the auxin was transported to the positive pole, which is theoretically in the direction expected of a dissociated acid. His experiments are to be severely criticized in that he used too few test plants. Usually 12 test plants give an average result correct within 10 per cent (see text below). Individual differences between two plants may be considerable.

The writer has reinvestigated the problem of electrical transport of auxin. Synthetic heteroauxin (indole-3-acetic acid) prepared in the Gates chemistry laboratories was used. Since this substance is a weak acid, it dissociates into a large anion and a hydrogen ion. The anion should be transported in an electric field to the anode. That heteroauxin conducts a current can be determined by actual conductance measurements.

In order to demonstrate that the heteroauxin used in the experiments about to be described actually conducts a current, conductance measurements were made so that the transport number and absolute mobility under unit potential gradient could be ascertained. The sodium salt of indole-3-acetic acid was prepared by accurately neutralizing the acid and recrystallizing from absolute alcohol. (The salt preparation was made by Dr. KOEPFLI of the California Institute of Technology.) The salt was then made up in different concentrations and their conductances determined in a calibrated conductance chamber at 25° C., using a conductance bridge with a Vreeland oscillator at 1000 cycles. The cube root of the concentrations was plotted against the equivalent conductances. This curve was extrapolated to the limiting conductance at infinite dilution. Since the limiting conductances of the hydrogen and of the sodium ions at 25° C. are known, the limiting

conductance of the acid could be determined from that of the experimentally determined conductance of the more dissociated salt. Knowing the limiting conductance of the acid, the dissociation constant could be calculated in order to check the accuracy of the conductance measurements. The dissociation constant is known to be about 2.0×10^{-5} at 25° C. (**15**, **7**). From the limiting conductance of the heteroauxin anion, its transport number and ionic mobility could be calculated.

Two experiments gave limiting conductances of the salt of 79.5 and 78.5, average 79.0.

Since Λ_o Na at 25° C. = 50.9 (NOYES and SHERRILL, **17**),
and Λ_o H at 25° C. = 350 (*ibid.*);
hence Λ_o auxin anion = 79.0 − 50.9 = 28.1,
and Λ_o auxin = 28.1 + 350 = 378 at 25° C.

Since the dissociation constant, K. is proportional to the degree of dissociation; hence

$$K = \frac{C \cdot a^2}{1 - a}, \text{ where } a = \frac{\Lambda}{\Lambda_0}, \ \Lambda =$$

the conductance at different concentrations, and C = the concentration in mols. For three concentrations used, the dissociation constants were:

Normality	$K \times 10^{-5}$
0.004	3.0
0.008	2.8
0.01	2.8
	Av. 2.9

For a comparison, KÖGL and KOSTERMAN'S values for the dissociation at 25° C., their determinations being based on pH measurements, are given:

Normality	$K \times 10^{-5}$
0.001	2
0.005	3
0.01	1
	Av. 2

DOLK and THIMANN (**7**) determined the dissociation constant of heteroauxin from *Rhizopus suinus* by partition coefficients and the dissociation at various pH's. Their average value (at room temperature) was 1.82, hence approximately that of KÖGL and KOSTERMANS.

It is seen therefore that the conductance measurements were quite accurate, and in fact gave better constants than either KÖGL and KOSTERMANS' or DOLK and THIMANN'S.

The transport number of the heteroauxin anion is equal to the ratio of the limiting conductances of the anion to that of the sum of the anion and cation:

$$28.1/378.1 = 0.07$$

Hence only 0.07 of an electric current is carried by the heteroauxin anion in solution. The absolute mobility of the anion under unit potential gradient of 1 v. per centimeter in a cube 1 cm. on each side is:

$$28.1/96{,}500 = 2.9 \times 10^{-4} \text{ cm./sec.}$$
$$= 10.5 \text{ mm./hr.}$$

Before experimenting with applied P.D.'s in plants, it was of interest to see how much of a potential gradient is necessary to establish a *measurable* difference in transport in a liquid or agar medium. The transport of heteroauxin to the anode can be obtained if a sufficiently high P.D. is used. The following experiments demonstrate that the electrolytic migration might easily escape observation unless the proper precautions are taken.

The following experiment shows a typical negative result:

Tap-water agar blocks $11 \times 8 \times 1$ mm. were soaked one hour in heteroauxin of a known concentration. One hour of soaking permitted complete equilibrium between block and solution. One of the blocks was then placed atop three tap-water blocks containing no auxin, and Ag-AgCl electrodes (freshly plated) applied at each end of the stack of blocks. A P.D. of 300 mv. (millivolts) was applied for one hour such that a 9 microampere current flowed. Another stack of blocks was set up in the same way, but with the polarity of the applied P.D. reversed. A control stack was run in which no current was passed. At the end of the hour, the blocks were removed, separated, and each one cut into 12 smaller blocks and tested for their auxin content by the standard *Avena* test.[1]

Five experiments of the kind just described were performed, and table I records typical results.

In another experiment, a P.D. of 450 mv. applied for one hour, at 28 microamp. of current-flow gave considerable electrolytic destruction of auxin. The concentration used in the top blocks was 20°. At the end of one hour only about 5° remained in all four blocks. The blocks nearest the anode

[1] The technique involves placing a small agar block containing auxin on one side of the stump of a decapitated *Avena* coleoptile so that the auxin from the block will diffuse down only along the side under the agar block, thereby causing this side to grow more than the other side. This will result in a curvature away from the side on which the block was placed. The curvature is proportional to the concentration of auxin in the block, to the size of the block, to the time between decapitation and placing the block, and to the time that the block is left on. The method is so controlled (temperature, humidity, light, tools, time, plants, etc.) that within certain limits of auxin concentration, the curvatures in degrees give a quantitative analysis of the concentration such that 1° curvature per 10-mm.^{3} block corresponds to about 10^{-7} to 10^{-9} mg. of heteroauxin. (This again depends upon the methods used and the sensitivity of the plants.) Usually 12 to 24 test plants are used for each determination, the averages being taken. The accuracy is about 10 per cent. when 12 plants are used. For detailed descriptions of the method, and other test methods, see WENT and THIMANN (28).

TABLE I

EFFECTS OF APPLIED P.D. ON AUXIN DIFFUSION OF AGAR AT LOW VOLTAGE
P.D., 300 MV.; CURRENT-FLOW,* 9 MICROAMP.; TRANSPORT TIME, 1 HR.

BLOCKS	DEGREES OF CURVATURE (AUXIN CONCENTRATION)		
	Control	Anode	Cathode
Top	12	9 ↑	10 ↓
	6	7	5
	5	2	4
Bottom	2	2	2
		Cathode	Anode
Total (sum)	25	20	21

* The arrows indicate the theoretical effect of the current flow on the auxin transport.

showed white discoloration which, on microscopic examination, proved to be due to minute gas bubbles, presumably of oxygen. When 10° auxin was used, and 600 mv. applied, complete destruction occurred.

The above experiments show clearly that when no electroosmosis or electrolytic destruction occurred, the applied P.D. had no effect on the transport of auxin in agar. Evidently higher voltages are necessary to effect a measurable change in transport. But at higher voltages, destruction by electrolysis occurs. Therefore it is easy to understand why DOLK obtained negative results.

Transport experiments in agar were then conducted, in which much higher voltages were used. Automatic siphons were used to wash away the products of electrolysis at the electrodes so that no auxin destruction might occur. Zinc electrodes were used, and the solutions at the electrodes were separated from the agar blocks by agar-gelatin seals. A 25° auxin solution was used to wash the top electrode. Analysis of the four blocks used in the experiment is given in table II.

TABLE II

P.D., 22.5 V.; CURRENT-FLOW, 0.1 MA. (MILLIAMPERE); TRANSPORT TIME, 1 HR.
EFFECTS OF APPLIED P.D. ON AUXIN DIFFUSION AT HIGH VOLTAGE

BLOCKS	DEGREES OF CURVATURE (AUXIN CONCENTRATION)		
	Control	Cathode	Anode
Top	6	26 ↓	0 ↑
	3	24	0
	2	19	0
Bottom	1	12	0
		Anode	Cathode

The control shows that transport by diffusion was much less than electrolytic transport. This P.D. represented approximately a 50-volt-per-centimeter gradient, and definitely causes auxin to be electrolytically transported to the positive pole. The results are not qualitatively distorted by electro-osmotic-flow, since water travels to the negative pole in agar and would only tend to decrease the amount of auxin carried by electrolysis to the positive pole.[2]

Electrical polarity and polar transport in *Avena* coleoptile

It has been demonstrated that sections of coleoptiles exhibit an electrical polarity which is in the direction expected by Went's theory of electrical transport (*cf.*, **5**), *i.e.*, the morphological apices are electronegative to the bases, and the negatively charged ion of the acid, auxin, could, then, theoretically be electrically transported polarly from tip to base. The following section is concerned with experiments attempting to directly demonstrate that polarity of auxin transport varies in the same way that electrical polarity varies when it is altered.

Transport technique

The transport experiments were performed as described by van der Weij (**25**), except that no holders were used to support the coleoptile sections, as this causes auxin leakage through water films in the holder. The seedlings were grown in sand from hulled seeds of *Avena sativa* (Victory oats) of a pure line stock (also used in the *Avena* tests). Germination, growth, transport experiments, and tests were all carried out in the experimental dark room at a relative humidity of 90 per cent., at 24° C., and in orange light not causing phototropism. The lengths of the sections used were in all cases 3 mm. They were cut from the subapical zones of coleoptiles 3 to 4 cm. in length, one to two sections per coleoptile. The section-cutters were made of two parallel razor blades separated by a brass strip. The sections were then placed in an upright position on wet filter paper by means of eye-forceps. This washes out enzymes which destroy auxin (**18**). After an hour of such "washing," the sections were removed, and excess moisture carefully removed from the sections with filter paper. Twenty sections were each then placed upright on the $11 \times 8 \times 1$-mm. agar blocks (tap-water agar) and another block containing the auxin placed on top of the sections. The sections were handled with fine eye-forceps. Agar blocks can be made to contain auxin in any desired concentration by soaking them in auxin solutions of known concentration for about an hour. The

[2] That water is carried to the negative pole was easily demonstrated by applying a P.D. of 1 or 2 v. across an agar thread 20×1 mm. The thread immediately swelled at the negative pole, and shrunk at the positive pole. Therefore, in the above experiment, auxin traveled to the positive pole *despite* any flow of water in the opposite direction.

transport time was usually one to two hours, depending upon the concentration of auxin in the top block. After this time, the two blocks were removed, cut into 12 smaller blocks, and tested on *Avena*. The sum of the values obtained for each block should approximate the original amount in the top block. The amounts of auxin transported were expressed as the percentages of the sums of the concentrations in top and bottom blocks present in the bottom blocks.

SHUNTING EXPERIMENTS.—ROSENE (**22**) demonstrated that the P.D. between apex and base of an *Allium cepa* root could be reversibly decreased by liquid shunts interposed between the contacts, and at a distance from them. On the basis that the P.D. between the two ends of a coleoptile section could be lowered by a shunt, it was thought that auxin transport might be simultaneously lowered. Hence metallic shunts (metal foil) were made between tops and bottoms of such sections, and transport experiments run. In the set-up of the experiment clean tin-foil connected the top agar block with the bottom agar block. Tin-foil not connecting the blocks was applied to the blocks of the controls. The results of a typical experiment are given in table III.

TABLE III

EFFECTS OF SHUNTING THE P.D. ON TRANSPORT OF AUXIN

TRANSPORT TIME, 1 HR.; LENGTH OF SECTIONS, 3 MM.; 20 SECTIONS PER EXPERIMENT

BLOCKS	DEGREE OF CURVATURE (AUXIN CONCENTRATION)	
	Shunted	Control
Top	8	8
Bottom	3	4.5
Total (sum.)	11	12.5
	Percentage of total curvature (sum)	
	%	%
Bottom	27	36

The difference between the shunted and the control experiments, – 9 per cent., is well within the experimental error.

In five similar experiments the differences in percentage of curvature (expressed as the above result, – 9 per cent.) were 12, 9, – 7, – 5, and – 2 per cent., with an average of 1.4 per cent., which is within the experimental error.

The results of the experiments showed that shunting the P.D. between the two ends of a section has no effect on the auxin transport. It is realized that such a shunt is probably very ineffective in reducing the individual

P.D.'s probably maintained across each cell. The electrical resistance of the coleoptile section is very high (10^5 ohms for 20 3-mm. sections in parallel), and the P.D. between the two ends is relatively very minute (a few millivolts). Hence any current dissipated by metallic shunts between the two cut ends would be extremely small, and the individual cell P.D.'s would be relatively unaffected. If the resistance of the tissue had been quite small, and there were relatively few cells between the electrodes, the situation might have been quite different (**8**). In the work of FRANCIS (**8**) the P.D. across a frog's skin was shunted through a total resistance of 1500 ohms, and the current-flow was measured. If the external resistance had been lower, the transport of ions across the skin might conceivably have been altered.

APPLIED P.D.'S AND TRANSPORT IN COLEOPTILE SECTIONS

KÖGL (**13, 14**) claimed to have found an effect of applied potentials on longitudinal transport in *Avena* coleoptiles, produced by placing agar blocks containing auxin on one side of decapitated coleoptiles and applying a P.D. between this block and the base of the plant. The curvatures resulting were increased when the tip was made the cathode, and decreased over the controls when it was made the anode. The auxin anion was presumably transported in the plant toward the anode, and prevented from being transported downward when the base was made the cathode. P.D.'s of 0.2 to 1.4 v., and currents of 0.4 to 2.8 microamp. were used. Later, however, KÖGL, HAAGEN-SMIT, and VAN HULSSEN (**16**), stated that the applied currents merely caused the auxin to be transported more rapidly from the agar into the plant, and that the effect was not on the longitudinal transport in the plant.

The effect of electric fields on growth of plants has been reviewed by WENT (**27**), RAMSHORN (**20**), and STERN (**24**). Even if some of the results could have been explained by the effects of electric currents on the transport of auxin in the plants, the results are conflicting, to the extent that no agreement has been reached that direct currents of one polarity affect growth in a definite way.[3]

Since it had not yet been shown that auxin will be longitudinally transported in plants under the influence of an electric current, it was important to obtain experimental evidence to this effect.

Transport experiments, such as described in the previous section, were performed while a P.D. was applied between the two blocks in such a way that the electrical current would theoretically augment transport in one case, and inhibit it in the other. Ag : AgCl electrodes were applied directly to the agar blocks, and P.D.'s of 25 to 750 mv. applied for 1 to 2 hours. The

[3] A paper by CHOLODNY and SANKEWITSCH in the April no. 1937, PLANT PHYSIOLOGY, describes the use of applied currents which were found to increase coleoptile growth if the apex is made the cathode, and to inhibit it if the apex is made the anode. Immediately after cutting off the current, however, inhibition ensued.

electrodes were the same size as the agar blocks, and were replated before every experiment. In a typical experiment heteroauxin concentration was 40° in the top block at the start. After one hour transport time, the top blocks were diluted 3X for analysis. The results of this experiment are seen in table IV.

TABLE IV

EFFECTS OF APPLIED P.D.'S AND TRANSPORT OF AUXIN IN COLEOPTILE SECTIONS
P.D., 300 MV.; TRANSPORT AND CURRENT-FLOW, 1 HR.; 20 3-MM. SECTIONS PER EXPERIMENT

BLOCKS	DEGREES OF CURVATURE (AUXIN CONCENTRATION)		
	Control	Anode	Cathode
Top	35	31 ↑	31 ↓
Bottom	8	7	7
		Cathode	Anode
Total (sum.)	43	38	38
	Percentage of total curvature (sum)		
	%	%	%
Bottom	18.6	18.4	18.4

In twelve experiments like the preceding in which the applied P.D. varied from 25 to 300 mv., the total average percentage of the sum of auxin concentration expressed as percentage of total curvature in the bottom block is shown in table V.

TABLE V

EFFECTS OF APPLIED P.D.'S AND TRANSPORT OF AUXINS IN COLEOPTILE SECTIONS

BLOCKS	PERCENTAGE OF TOTAL CURVATURE (SUM) (AUXIN CONCENTRATION)		
Top	Control	Anode	Cathode
	%	%	%
	25	21 ↑	20 ↓
Bottom	.....	Cathode	Anode

It is obvious that no effects of applied P.D.'s on transport were found.

Since no results were obtained in the above experiments, it was thought that perhaps the potential gradients were not steep enough. The absolute mobility of the heteroauxin anion is 10.5 mm. per hour at 1 v. per centimeter. We should, therefore, expect 300 mv. per 3 mm., which is nearly 1 v. per centimeter, to suffice, regardless of the fact that the conducting path in the experiments just described is not exactly like the standard cube usually

referred to when the term, "absolute mobility," is used. It was found, however, in the experiments using Ag, AgCl electrodes, that a potential difference of more than 300 mv. caused destruction of auxin. It must be remembered that hydrogen ions, and ions of other salts to be found in agar and tap water, will carry the current, the auxin carrying only 7 per cent. of the current. Hence it would be necessary to apply larger currents for longer periods of time, especially when high resistances are encountered (*e.g.*, 50 v. per cm. were necessary in the case of agar).

Since electrolytic destruction of auxin occurred when P.D.'s above 300 mv. were used, recourse to flowing electrodes was taken. The same type of siphon-flow electrode vessels were used as were described in the section on transport in agar, except that the apparatus was made so that the electrode vessel and top-agar block could be racked down to the top of the sections placed on the bottom block, by means of a set-screw mechanism. This dispensed with clumsy manipulation and the danger of upsetting the sections, or of injuring them by loading them down with excess weight. The fluid used for washing the top electrode was 0.1 N KCl with auxin, and for washing the bottom electrode 0.1 N KCl without auxin. Current was passed so as to theoretically augment transport in one case, and to decrease it in the other (opposite polarity). No difference was found, however, in the amounts of auxin transported, even when P.D.'s of 3 v., and a current of 0.04 ma. was used. The sections, after current treatment, were "washed" in an upright position on wet filter paper, and tested in further transport experiments to see if there were any after-effects of the current treatment, but none were observed. It was not easy to wash out the auxin remaining in the sections left there by the first treatment, so that any after-effects occurring might be masked by contamination. Hence sections were current-treated in the apparatus *without* auxin application, and then immediately tested in transport experiments for any changes in their ability to transport normally. A description of part of the set-up of a typical experiment to determine the after-effect of applied P.D. on transport follows. Forty 3-mm. sections from subapical zones of coleoptiles were used for each experiment, *i.e.*, for each polarity of the applied P.D., and for the control. Each lot of 40 sections in each experiment was then halved for duplicate transport experiments, using an auxin concentration of 15° in the top blocks. The average results of each duplicate set are shown in table VI.

The results of the average of four experiments like the preceding are given in table VII as the percentage of the total amount of auxin in top and bottom present in the bottom blocks. These results signify no effect of the current on transport in the sections. Higher currents, *i.e.*, above 1 ma., caused the sections to become flaccid, and hence could not be used in any of the transport experiments, as was found necessary in the case of agar.

TABLE VI

AFTER-EFFECTS OF APPLIED P.D. ON TRANSPORT OF AUXIN

P.D., 1.5 v.; CURRENT-FLOW, 0.8 MA.; CURRENT TREATMENT 1 HR.; TRANSPORT-TIME 1 HR.

BLOCKS	DEGREES OF CURVATURE		
	Control	Anode	Cathode
Top	7.2	7	7
Bottom	6	5 ↑	4.6 ↓
		Cathode	Anode
Total (sum.)	13.2	12	11.6
	Percentage of total curvature (sum)		
	%	%	%
Bottom	45	42	40

TABLE VII

PERCENTAGE OF AUXIN IN BOTTOM BLOCKS

BLOCKS	PERCENTAGE OF TOTAL CURVATURE (SUM)		
Top	Control	Anode	Cathode
	%	%	%
	38	37 ↑	39 ↓
Bottom	.....	Cathode	Anode

EFFECT OF APPLIED P.D.'S ON INHERENT P.D.'S AND TRANSPORT IN *AVENA* COLEOPTILE SECTIONS

The question now arose: Does the inherent P.D. of the sections change appreciably when such P.D. applications are made? To answer this question, transport experiments were performed in which the transport, inherent and applied P.D.'s, were measured simultaneously.

Several such experiments were performed, and the following was a typical setup. Three-mm. sections were cut and washed in an upright position for 1 hr. on wet filter paper. Twenty such sections were placed on tap-water agar blocks in a moist chamber, electrical contact being made to the block with a strip of 0.1 N KCl agar which led through paraffined glass tubes outside the moist chamber to a cup filled with 0.1 N KCl in which the Zn: $ZnSO_4$ electrode could be placed. The top agar block containing auxin had a similar KCl-agar strip leading to another cup and electrode outside the chamber. The control transport experiment had KCl-agar strips of the same size contacting the top and bottom blocks. The inherent P.D.'s of such a setup were measured with the string electrometer, as well as the applied P.D.'s. The current was measured with a microammeter. It was found

that the electrodes did not polarize with the currents used, so that the same electrodes used for applying the P.D.'s could be used for measuring the change in inherent P.D.'s of the sections. In such a transport experiment, it is seen that the P.D.'s of 20 sections in parallel are measured, thus the average. P.D.'s were measured and applied, cut off after a time, and the inherent P.D.'s immediately measured. About 2 seconds elapsed between two such readings, but it was observed that the inherent P.D.'s maintained their levels several seconds after current treatment of a minute or so. In other words, depolarization was not so rapid that the effect of the applied P.D.'s on the inherent P.D.'s could not be accurately determined with the short-period string electrometer.

The transport time was 90 minutes; the applied P.D., 3 v.; and the current-flow, 10 microamp. *Before* the P.D. was applied, the inherent P.D.'s were: Cathode at the top (current flow theoretically augmenting transport), apex 2 mv. negative to the base; anode at the top (current theoretically inhibiting transport), apex 4 mv. negative to the base. Hence the same polarity existed as was found in an earlier part of this paper. 5 minutes after the current was turned on, the inherent P.D. was measured, after briefly cutting off the applied current. Every 5 minutes thereafter during the course of the experiment, the inherent P.D. was measured and recorded, and found to be the same throughout, namely: Cathode at the top (applied polarity same as inherent), apex 43 mv. negative to base; anode at the top (applied polarity opposite inherent polarity), 55 mv. *positive* to base. The blocks were analyzed for their auxin content at the end of the 90 minutes. The results obtained are given in tables VIII and IX.

TABLE VIII

EFFECT OF APPLIED P.D.'S ON INHERENT P.D.'S AND TRANSPORT IN *AVENA* COLEOPTILE SECTIONS

INHERENT APICAL NEGATIVITY INCREASED FROM 2 TO 43 MV.

BLOCKS	DEGREE OF CURVATURE (AUXIN CONCENTRATION)	
	Control	Cathode
Top	4	3
Bottom	5	4
		Anode
Total (sum.)	9	7
	Percentage of total curvature (sum)	
	%	%
Bottom	56	57

TABLE IX

EFFECT OF APPLIED P.D.'S ON INHERENT P.D.'S AND TRANSPORT IN *AVENA* COLEOPTILE SECTIONS

INHERENT POLARITY INVERTED FROM APICAL NEGATIVITY OF 4 MV. TO A POSITIVITY OF 55 MV.

BLOCKS	DEGREE OF CURVATURE (AUXIN CONCENTRATION)	
	Control	Anode
Top	4	4 ↑
Bottom	3	3
		Cathode
Total (sum.)	7	7
	Percentage of total curvature (sum)	
Bottom	43	43

Five experiments like the preceding gave the same result, namely, that inverting or increasing the inherent electrical polarity had no effect on the polar transport.

GEOELECTRICAL POLARITY AND POLAR TRANSPORT OF AUXIN

WENT (**26**), VAN DER WEIJ (**25**), and PFAELTZER (**19**) have clearly demonstrated that gravity has no effect on the polar transport of auxin in *Avena* coleoptile sections. It has been shown in a recent paper by the writer (**5**) that inverting sections inverts their electrical polarity for a time. Should it be shown that this inverted polarity, during the time that it exists, has no effect on polar transport, the evidence would be stronger against the thesis of electrical transport. To this end, transport experiments were performed, in which sections were inverted, and the inherent electrical polarity and transport of auxin were measured. Figure 1 gives the results of such an experiment. The setup of this experiment was as follows:

Sections were cut and divided into two lots. One lot was placed on wet filter paper in an upright position; the other lot was placed in an inverted position. After 1 hour, 20 sections from each lot were removed and a 90-minute transport experiment run on each lot, the sections remaining in the position in which they had been placed on the filter paper. One auxin agar block was placed on the inverted apices of the inverted lot, and another on the upright apices of the upright lot. During the transport, the P.D. between each block of the two transport experiments was measured several times during the experiment. The curves in figure 1 represent the change in these P.D.'s with time. At the end of two hours, two more lots were removed from the wet filter paper and the process repeated, as was also done for two more lots left standing 4 hours after sectioning. In this way, the

effect of gravity could be followed over a period of several hours. Figure 1 contains the results for the upright and inverted sections left in those respective positions in transport experiments for 1, 2, and 4 hours, thus including six experiments. The P.D.-time curves of each of the six experiments; and diagrams representing the auxin analyses of the top and bottom blocks, together with the tabulated sums of the top and bottom blocks, and the percentage of this sum in the bottom blocks, are also shown in figure 1.

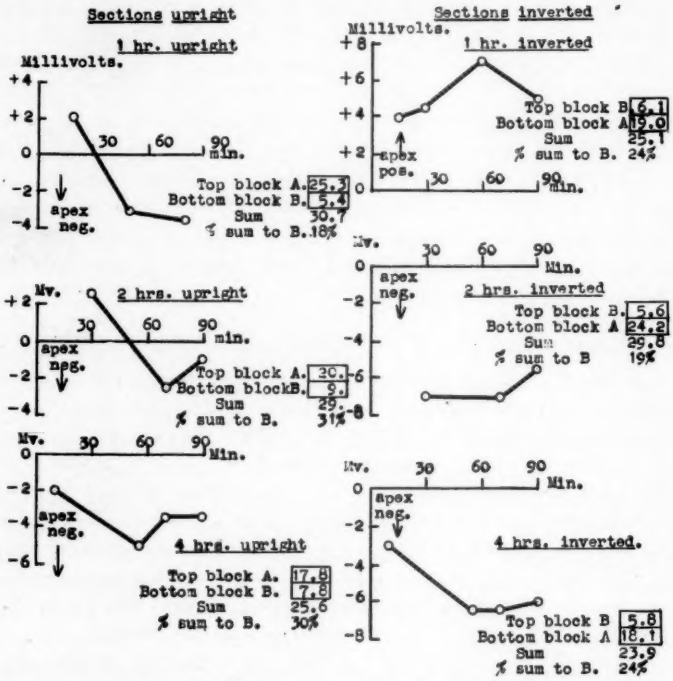

FIG. 1. Effect of inverting on electrical polarity and auxin transport.

EFFECT OF INVERTING ON ELECTRICAL POLARITY AND AUXIN TRANSPORT.— In the case of the upright sections, the P.D.-time curve shows the usual characteristics, namely, that time is necessary before the apical negativity is established, in this case less than an hour (5); and, in the case of the sections left standing 4 hours, this polarity was already established. It is seen that the sections inverted one hour show the usual inverted electrical polarity, *i.e.*, apical positivity, and that this positivity is maintained throughout the transport experiment; hence if the electrical transport theory were correct, the *transport* polarity should also be inverted. After two hours of

inversion, the inverted sections have regained their normal polarity, a characteristic already discussed in the foregoing pages. After 4 hours, the normal polarity was even more pronounced.

It is seen at once that regardless of the fact that the electrical polarity was inverted, in the case of the sections left inverted one hour, the amount of auxin transported is the same as in the upright section with normal polarity. In all cases in figure 1, the differences (in percentages of the sums of top and bottom blocks found in the bottom blocks) between transport experiments on upright and inverted sections were within the limits of error.

The results reported here clearly demonstrate a close parallelism between the known direction of auxin transport inside the plant and the *normally* existing electrical polarity described in the writer's recent paper (**5**), namely: that the tip of the coleoptile of *Avena* is electronegative to the base; and the dissociated anion of heteroauxin could be electrolytically polarly transported to the electropositive base. That auxin is polarly transported has also been shown by evidence discussed in the introduction to the writer's recent paper (**5**). It was then shown that this electrical polarity could be changed and inverted by means of applied electromotive forces, and by gravity (and also by light, as was shown in an earlier paper, (**4**)); while the amount and polarity of auxin transport remains the same. These facts were taken to mean that the polarity of auxin transport and electrical polarity are independent of one another.

The works of BRAUNER and BÜNNING (**3**), KOCH (**12**), and AMLONG (**1**) have shown that the lateral transport of auxin in plant organs is probably linked with geo- and photoelectric P.D.'s, or at least have shown a close relationship between the two (**5**). These P.D.'s were measured in approximately the same way as described above. Moreover, light, gravity, and applied P.D.'s can cause tropisms. Light and gravity induce lateral transport of auxin. Applied P.D.'s may also induce lateral transport, as indicated by electrotropisms, but this is not as yet directly proved.[4] Since light, gravity, and applied P.D.'s do not affect longitudinal transport, it is probable that lateral and longitudinal transport are caused by two entirely unrelated sets of factors.

It is, then, obvious that the P.D.'s measured and discussed in this paper as comprising the plant's electrical polarity are not the cause of polar auxin

[4] BRAUNER and BÜNNING (**3**) reproduced photographs of electrotropisms in *Avena*. The curvatures are in no way similar to those in photo- or geotropisms, and do not suggest a normal lateral transport of auxin. The applied P.D.'s might induce lateral transport of acid, which would increase the activity of auxin (**2**) on one side, thus causing the "electrotropism." AMLONG (**1**), however, indicated some linkage between auxin and electrotropisms, as decapitated roots of *Vicia faba* and decapitated seedlings exhibited no electrotropisms. SATUNSKIJ (C. R. Acad. Sci. U.R.S.S.; n.s. 2: 295–298. 1936) presents additional evidence indicating that auxin might be laterally displaced to the induced anode of coleoptiles in an electrostatic field.

transport. It is still possible that polar transport is effected by a more subtle type of potential gradient not readily amenable to measurement by the methods described above and in a previous paper (5), and that such theoretical P.D.'s are located at phase boundaries or at capillary surfaces (electrokinetic potentials).[5] In the lack of evidence for the existence of the latter type of potential at phase boundaries, etc., in the plant, it is evident that electrical polarity, as described above, and the polar transport of auxin in plants are independent of each other.

Summary

1. Heteroauxin (indole-3-acetic acid) has a transport number of 0.07 and an absolute mobility of 10.5 mm. per hour under unit potential gradient of 1 v. per centimeter, as measured by conductance experiments.

2. Heteroauxin is electrolytically transported in agar to the anode in an electric field. A potential gradient of 50 v. per centimeter definitely influences the transport. Strictly non-polarizable conditions are necessary in order to establish such an influence.

3. Applied E.M.F.'s have no influence on the longitudinal transport of auxin in *Avena* coleoptiles, although these applied E.M.F.'s reverse or increase the inherent electrical polarity of the same sections.

4. Inverted electrical polarity induced by gravity has no effect on longitudinal auxin transport in coleoptile sections.

5. It is concluded that electrical polarity either has no cause and effect relation to the polarity of auxin transport in the *Avena* coleoptile, or that this relation is not amenable to treatment by the bioelectric methods outlined in this paper. The relation may be real but more subtle than has been revealed by the various types of electrometric measurements employed.

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[5] An example of such a potential gradient might be that in FÜRTH's (9) "Hochspannungsmodell," in which a potential gradient of several thousand volts per centimeter, produced at a stone electrode, affects dye-uptake in much the same way as tissues concentrate or accumulate substances (10), (11). Another example might be the phase boundary potentials measured by SCHULMAN and RIDEAL (23). These potentials are caused by orientation of polar molecules at interfaces and are measured by electrodes suspended in air above the interfaces, conductivity being produced by ionization of the air by polonium. RIDEAL, in a private communication in 1933, described how a current flow could occur at such interfaces, involving ionic transfer. The details of such mechanisms are discussed by RIDEAL (21).

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CARBOHYDRATE CHANGES WITHIN THE NEEDLES OF *PINUS PONDEROSA* AND *PSEUDOTSUGA TAXIFOLIA*

CLAIR L. WORLEY

(WITH FIVE FIGURES)

Introduction

The ability of most coniferous trees and of certain other plants to retain their foliage over several seasons has attracted considerable attention among botantists. This interest is especially keen concerning those plants subjected to extremely cold winters, and which, with rare exceptions, must possess the property of cold resistance, at least during the actual duration of the periods of subzero temperatures. It has long been known, even by the layman, that such coniferous needles are not, in general, resistant to cold during the warmer months of the year.

Consistent studies of the seasonal variations in the physiology of a plant, as well as parallel studies of the variations in the habitat-factors, are necessary to reach an understanding of that species' ability to survive under the environmental conditions representing a given habitat. It has long been recognized that the polysaccharides in leaves, under certain climatic conditions, are converted into the simpler saccharides, and vice versa, by what appears to be the shifting of an equilibrium by means of enzymatic activities. These seasonal variations in the amount and chemical nature of food reserves in the cells of evergreen leaves are in themselves of physiological significance. Although many workers have contributed much important data on this subject, including carbohydrate, osmotic pressure, and pH measurements, our actual information is still fragmentary.

This problem was undertaken to ascertain the quantitative fluctuations of various classes of carbohydrates in *Pinus ponderosa* and *Pseudotsuga taxifolia* needles throughout the more or less dormant season, and to determine a possible relationship between the saccharide fluctuations and the temperature changes.

Historical data

The measuring of fluctuations in amounts of carbohydrates has been investigated by numerous workers, the majority of whom have limited themselves to diurnal variations when studying photosynthetic tissues. MILLER's (**8**) measurements show that the non-reducing sugars in the leaves of most plants are in excess of the reducing sugars, and that the maximum point of increase of the total sugars is also the maximum point of increase of the non-reducing sugars. TOTTINGHAM *et al.* (**13**) found that the time of day

was less significant than temperature in determining carbohydrate composition; and that solar radiation is the limiting factor in the increases of nonreducing sugars when its value approaches 30° C. In addition to a comprehensive resumé on this phase, CLEMENTS (**2**) contributed several important pieces of research. He found that starch in the leaf of the sunflower can drop from the maximum to the minimum within one to two hours. The starch content increases after sunset and this, according to CLEMENTS, can be explained only on an actual return of sugars from the petioles. The data which represent the variations in the content of hemicelluloses were of tantamount significance; indicating, as it did, that the part played by these reserves in the metabolism of plants has been generally underestimated.

The seasonal fluctuations in carbohydrates has been investigated for the woody portions of many plants and especially for the apple and the sugar maple trees. There appears to be a quite general agreement among these investigators that starch accumulates in the phloem and the cortex, and perhaps the xylem, of trees in regions of severe winter climate; and in deciduous trees in regions of milder winter climate during the summer and autumn it diminishes to a minimum or disappears in winter, reappears in large quantities in the spring, and diminishes to another minimum as the buds are unfolding. They also found that a relationship exists between starch and sugars; namely, that as the one increases the other decreases, and vice versa. MURNEEK (**10**), TRAUB (**14**), HOOKER (**4**) and MURNEEK and LOGAN (**11**) have furnished the most important data for apple trees; while JONES and BRADLEE'S (**5**) paper is the classic and most recent investigation dealing with the sugar maple.

Some work has been completed on seasonal trends of carbohydrates in photosynthetic tissues. SPOEHR (**12**) in his classic observations on the seasonal variations of carbohydrates in *Opuntia phaeacantha* found that low water content and high temperatures are associated with an increase of pentosans, while a high water content and lower temperatures are associated with a decrease of polysaccharides, an increase of monsaccharides, and a decrease of pentosans. CAMERON (**1**), while investigating the orange tree, discovered that a different state of affairs occurs in evergreen trees in regions of mild winter climate. His research shows a constant increase in reserves during the autumn and winter. The maximum of reserves, as much as 25 per cent. of the fresh weight, is in the early spring just prior to blossoming. MIYAKE (**9**), LIDFROSS (**6**), and others have carried out microscopic observations and have reported that the majority of evergreen leaves of Japan and Europe nearly lost the starch from the mesophyll and the guard cells in winter. The starch content of Japanese coniferous needles is generally more abundant in spring than in late summer or early autumn.

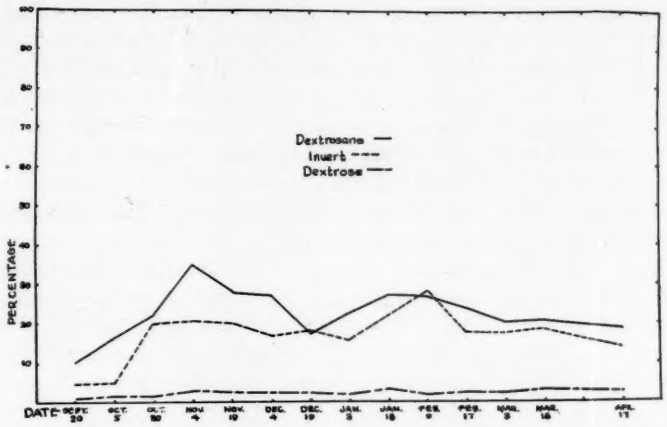

FIG. 1. Percentages of dextrose, invert sugar, and dextrosans occurring in *Pseudotsuga taxifolia* needles when based on fresh weights.

The literature has revealed but one paper closely related to the present investigation. MEYER (7) attempted to find a plausible correlation between physiological changes within the needles and the phenomenon of cold resistance in *Pinus rigida*. His data correspond to that found for the apple and the maple presented above, and, like them, he concluded that the autumnal accumulation of sugars probably results from temperature effects upon the equilibria between soluble and insoluble carbohydrates. This accumulation of sugars during the winter may also be important in the cold resistance of the pitch pine needles through the protective action which sugars exert against precipitation of proteins.

Material and methods

This study was conducted in the Plant Physiology Laboratory of the University of Idaho at Moscow. The material involved was collected from September, 1935 to May, 1936, and was obtained from the university arboretum immediately south of the campus.

Samples of the needles were taken every fifteen days, at daybreak, just before sunrise. This time of the day was chosen to reduce any variation due to diurnal fluctuations. Needles were always taken from the same trees, and always from the lower branches on the south side. This procedure was an endeavor to obviate, as nearly as possible, such factors as differences in the degree of sunlight, of moisture, and of wind, the relative juxtaposition of neighboring trees, and the individualism of different specimens of the same species.

The needles were brought into the laboratory intact with the branches. If they were moist from dew or rain, it was first necessary to dry them by rolling between paper towels. During the winter months frost, ice, and snow encrusted the needles; and, in such cases, the frozen layer was melted by a current of warm air and dried as above. Approximately 100 gm. of these whole needles were diced into pieces about one-eighth of an inch in length. The diced needles were weighed and recorded as the actual sample. The sample was placed in a wide-mouthed Erlenmeyer flask, in which 0.25 to 0.50 gm. of sodium carbonate had been added to an already boiling medium of 95 per cent. alcohol. Enough 95 per cent. alcohol was used to bring the final concentration of the alcohol to 80 per cent., assuming the fresh weight of the needles to be one-half water. The needles were boiled twenty or more minutes, were covered to a depth of an inch by adding additional 80 per cent. alcohol, and were then stoppered tightly until ready for use.

Owing to the usual difference in temperature outside and in the laboratory it was necessary to complete the above procedure as rapidly as possible to prevent ensuing chemical reactions from altering leaf material. By the method used, the enzymes in the plant tissues should have been almost instantly inactivated. The sodium carbonate, as well as the boiling alcohol, facilitated the destruction because of its alkaline nature and its rapid diffusion into the plant cells. The salt is important in another respect; namely, it neutralizes the various acids which might occur in the needles and which might hydrolyze complex carbohydrates and sugars.

Concomitantly, with the above sampling, determinations of the dry weights were conducted. Duplicate samples—10 to 15 gm.—of homogenous freshly diced needles, taken from the same branches as above, were subjected to 98° C. for two hours to inactivate the enzymes and were then transferred to a vacuum oven at 82° C. to complete the desiccation.

The alcoholic solution was filtered from the stored samples and the residue was washed several times with hot alcohol. The filtrate was again stoppered and stored, while the residue was placed in the vacuum oven to desiccate. This desiccated sample was cooled and weighed, and then ground to a powder fine enough to pass a 100-mesh sieve. Owing to its hygroscopic nature it was necessary to redesiccate in vacuo before weighing the ground residue. The difference in weights represented the amount lost due to grinding, and proved to be an essential factor for the computation of the total polysaccharides.

A Soxhlet extractor was employed to insure complete extraction of the various plant sugars in the ground residue. Seventy per cent. alcohol was used, and it was found, that with this concentration, two and one-half hours' extraction would insure complete removal of sugars from the material.

After extraction, the contents of the extraction flask were added to the original filtrate, and the thimble with its contents was replaced in the vacuum oven to remove all traces of absorbed alcohol. The combined extracts, to which 300 to 500 cc. of distilled water were added, were converted into an aqueous solution by distillation. This aqueous extract was cleared with neutral lead acetate and sodium oxalate. It was filtered into a liter volumetric flask and brought up to volume. The cleared solution was then taken for the determination of the alcohol-soluble free reducing substances. Twenty-five cc. of the cleared solution was used as an aliquot part, which not only insured easy computations but kept the measured amount within the range of available tables.

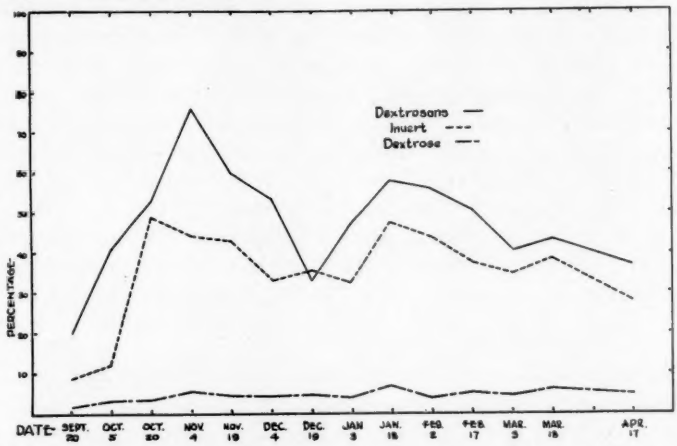

FIG. 2. Percentages of dextrose, invert sugar, and dextrosans occurring in *Pseudotsuga taxifolia* needles when based on dry weights.

To measure the non-reducing sugars (or better, the alcohol-soluble acid-hydrolyzable substances) a 100-cc. aliquot of the cleared solution was used. This aliquot was placed in a 250-cc. Erlenmeyer flask, and 10 cc. of concentrated HCl (s.g. 1.18) were added. Hydrolysis was allowed to proceed for twenty-four hours at room temperatures. At the end of twenty-four hours the acid was neutralized with Na_2CO_3 and filtered. The filtrate was put into a one-liter volumetric flask and made up to volume. An aliquot of 25 cc. was taken for reduction to determine the total alcohol-soluble free reducing substances plus the alcohol-soluble acid-hydrolyzable reducing substances.

The total polysaccharides, which included all the alcohol-insoluble acid-hydrolyzable reducing substances, were measured as follows: An aliquot part, approximately 3 gm., of the Soxhlet-thimble residue was used for

hydrolysis. The aliquot was placed in a 500-cc. flask and 100 cc. of water and 10 cc. of concentrated HCl (S.G. 1.18) were added. It was refluxed for two and one-half hours, neutralized, filtered into a liter volumetric flask and made up to volume. A liquid aliquot of 25 cc. was then taken for reduction.

The BERTRAND modification of the MUNSON and WALKER method was used exclusively for the determination of sugars in the above three solutions.

For reasons presented elsewhere in this paper the three classes of carbohydrates were called dextrose, invert sugar, and dextrosans respectively. The total weight of dextrose was computed by determining the amount of copper reduced in an aliquot of the original aqueous solution. In the second instance, the copper reduced was due to the additive powers of the alcohol-soluble free reducing substances and of the alcohol-soluble acid-hydrolyzable reducing substances. Consequently, it was first necessary to subtract the amount due to the dextrose from this total in order to compute the amount due to the acid-hydrolyzable compounds themselves. The computation of the total amount of polysaccharides was more complex, since more factors were involved. First, the amount of copper was transferred to grams of dextrose. Secondly, the weight of dextrose was multiplied by 0.9 to give the amount of dextrosans in each aliquot part. Thirdly, the following formula was employed to convert this into the total amount of polysaccharides in any given sample:

$$\frac{X : 40 \cdot R \cdot S}{H \cdot G} = P.$$

The figure and letters in the formula have the following significance:

X = amount of dextrosan per aliquot.
40 = the part each aliquot is of the liquid whole.
H = the amount of residue used for hydrolysis.
R = the amount of Soxhlet-extracted residue.
G = the weight of the ground residue.
S = the weight of the original alcohol residue, before grinding.
P = the total weight of polysaccharides in each specific sample.

Presentation and discussion of data

The chemical data presented herewith in tabular and graphic forms are grouped so as to show most conveniently and clearly the variations in chemical changes of the needles of *Pinus ponderosa* and *Pseudotsuga taxifolia* during the more or less dormant seasons of 1935 and 1936. The data presented portray certain specific tendencies of the carbohydrate metabolism within chlorenchymatous organs.

No qualitative tests were conducted on the three extracts, prior to measurements, to discover the specific reducing compounds concerned. It

seemed feasible to assume that the extracts of coniferous needles would yield reducing substances which were to a greater degree identical with like materials in similar extracts of other plants. Most investigators have like-

TABLE I

QUANTITATIVE TESTS FOR CARBOHYDRATES IN NEEDLES OF *PSEUDOTSUGA TAXIFOLIA*

DATE	GREEN WT.	DRY WT.	DEXTROSE PERCENTAGE OF		INVERT PERCENTAGE OF		DEXTROSANS PERCENTAGE OF	
			GREEN WT.	DRY WT.	GREEN WT.	DRY WT.	GREEN WT.	DRY WT.
	gm.	%	%	%	%	%	%	%
Sept. 20	94.589	49.523	0.91	1.83	4.36	8.80	9.91	20.02
Oct. 5	100.784	39.359	1.29	3.27	4.84	12.30	16.12	40.96
Oct. 20	99.018	40.896	1.53	3.74	19.92	48.70	21.81	53.33
Nov. 4	104.159	46.353	2.68	5.78	20.55	44.32	35.28	76.11
Nov. 19	93.767	46.072	2.25	4.88	19.80	42.96	27.47	59.61
Dec. 4	99.787	50.309	2.17	4.32	16.64	33.07	27.00	53.66
Dec. 19	99.417	50.529	2.32	4.59	17.99	35.59	16.71	33.06
Jan. 3	99.015	47.317	1.94	4.11	13.39	32.53	22.28	47.08
Jan. 18	97.177	46.504	3.21	6.90	22.10	47.53	27.02	58.09
Feb. 2	99.868	47.329	1.89	4.00	20.79	43.92	26.62	56.24
Feb. 17	101.604	46.633	2.55	5.46	17.53	37.57	23.76	50.96
Mar. 3	99.801	49.500	2.31	4.66	17.31	34.98	19.99	40.38
Mar. 18	101.587	47.377	3.04	6.41	18.27	38.56	20.59	43.25
Apr. 2 (No samples)	.....	.....	.....	.....	.....	.....	.....	.....
Apr. 17	99.984	50.583	2.49	4.93	14.08	27.84	18.68	36.83

TABLE II

QAUNTITATIVE TESTS FOR CARBOHYDRATES IN NEEDLES OF *PINUS PONDEROSA*

DATE	GREEN WT.	DRY WT.	DEXTROSE PERCENTAGE OF		INVERT PERCENTAGE OF		DEXTROSANS PERCENTAGE OF	
			GREEN WT.	DRY WT.	GREEN WT.	DRY WT.	GREEN WT.	DRY WT.
	gm.	%	%	%	%	%	%	%
Sept. 20	100.410	42.067	0.87	2.07	3.55	8.43	11.69	27.79
Oct. 5	108.583	41.045	0.99	2.41	3.06	7.45	19.43	47.33
Oct. 20	91.127	41.643	2.36	5.66	12.03	36.36	20.20	48.51
Nov. 4	97.237	43.236	2.08	4.81	21.93	50.72	29.71	68.72
Nov. 19	102.602	45.790	2.20	4.81	19.26	42.06	26.53	57.94
Dec. 4	99.638	45.369	2.09	4.61	15.53	34.24	26.31	57.98
Dec. 19	99.844	44.863	2.29	5.11	17.27	38.49	20.55	45.80
Jan. 3	98.740	43.497	2.21	5.09	21.55	49.55	23.56	54.16
Jan. 18	100.472	43.322	2.10	4.85	15.33	35.38	25.67	59.25
Feb. 2	100.189	42.417	2.05	4.83	19.40	45.74	23.89	56.31
Feb. 17	101.634	41.490	2.59	6.25	20.37	49.09	28.17	67.89
Mar. 3	99.715	42.629	3.01	7.07	16.41	38.49	24.05	56.41
Mar. 18	100.752	44.221	2.85	6.45	22.35	50.55	32.66	73.85
Apr. 2 (No samples)	.....	.....	.....	.....	.....	.....	.....	.....
Apr. 17	101.845	44.690	1.65	3.70	12.92	29.45	19.98	45.53

wise assumed that the reducing substances were various sugars without first conducting a qualitative test to authenticate their assumption. This has been a successful assumption to date; however, it leads into an impossibility in the present investigation. Tables I and II show that on November 4, 1935, as well as for other days, the total carbohydrates exceeded the dry weight. Since all previous papers have followed this nomenclature and since it simplifies the phraseology of the discussion, the terms dextrose, invert, and dextrosans will be used, meaning respectively, the alcohol-soluble free reducing substances, the alcohol-soluble acid-hydrolysable reducing substances, and the alcohol-insoluble acid-hydrolysable reducing substances.

It has long been known that a marked difference between gymosperms and angiosperms occurs not only in the structure but also in the chemical composition of the tissues. The cell sap of conifers has yielded pentose sugars in considerable quantity, and, likewise, the hydrolysis of their cell walls has shown that pentosans constitute the major portion of the structural hemicelluloses. A qualitative test was conducted for pentose sugars and pentosans on November 4 and succeeding dates. Positive results were attained for the dextrose and dextrosan extracts. Their decomposition product, furfural, was detected in both the invert and the dextrosan extracts. Coniferous trees synthesize oil with the approach of cold weather

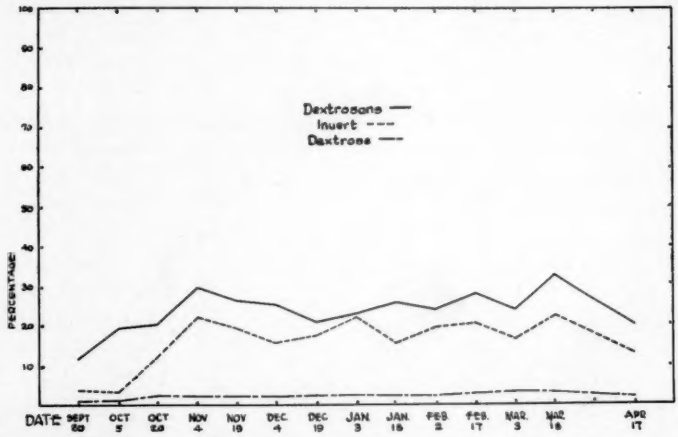

FIG. 3. Percentages of dextrose, invert sugar, and dextrosans occurring in *Pinus ponderosa* needles when based on fresh weights.

in such noticeable quantities that the Germans have frequently referred to them as the "Fettbäume." During this synthesis, hexose sugars are changed into precursors of oils, gums, and fats via pyruvaldehyde and pyruvic acid, both of which will reduce Fehling's solution. If either or both

of these substances were known to be present, or if compounds were known to be present which would on hydrolysis yield these reducing substances, the high amount of cuprous oxide released could be explained. No qualitative tests were conducted for these compounds because of the complicated and lengthy procedures involved. There remains one other plausible explanation; namely, that certain other decomposition compounds with reducing properties occur in the extracts. That this last actually did occur has been partially proven. Formaldehyde was detected in the dextrosan extract on November 4 for both species. Secondly, the sudden drop in temperature the first few days of November, prior to the establishment of the needles' cold-resisting mechanism, injured the needles of conifers throughout the arboretum. They became brown, flaccid, and underwent abnormal senescence. Some rotted on the limbs, while others dropped throughout the winter. This specific condition is abnormal to prevailing conditions in the Pacific Northwest. Although needles with macroscopic injuries were avoided, it does not prove that the samples were void of material with internal injuries and decomposition products.

Repeated checks have definitely proven that the fallacy is not in the procedure and methods involved but must be incorporated in the interpretation of the data secured. The various extracts, and especially the dextrosan, must therefore contain other reducing compounds in addition to the sugars.

Considerable care should be taken before drawing conclusions from the data based on percentages. The order of error, under the conditions here measured, seems about the same whether expressed as percentage of dry weight or of fresh weight if, on the one hand, the change is due to dry matter loss, and, on the other, to water absorption.

Attention is called to the low percentages and the limited variations in the dextrose during the colder seasons. Figures 1 and 3 illustrate this uniformity, which would be even more striking if based on its divergence from a seasonal average. That dextrose remains a fairly uniform constituent part of the coniferous needles is in accord with results obtained by JONES and BRADLEE (**5**) and others. These results, however, are not universal, for MURNEEK and LOGAN (**11**), HOOKER (**4**), and TRAUB (**14**), all of whom worked with the apple, found significant variations of these reducing sugars throughout the year's cycle; however, even they found a marked uniformity during the colder seasons. MEYER'S (**7**) results on the pitch pine leaves showed a limited variation in dextrose from October to April. All of the above data point to the lability of dextrose solutions, which indicates that it might be an intermediate product involved in the metabolism of the plant. Undoubtedly, upon reaching a certain maximum percentage, dextrose is condensed or polymerized into reserves and structural materials of the

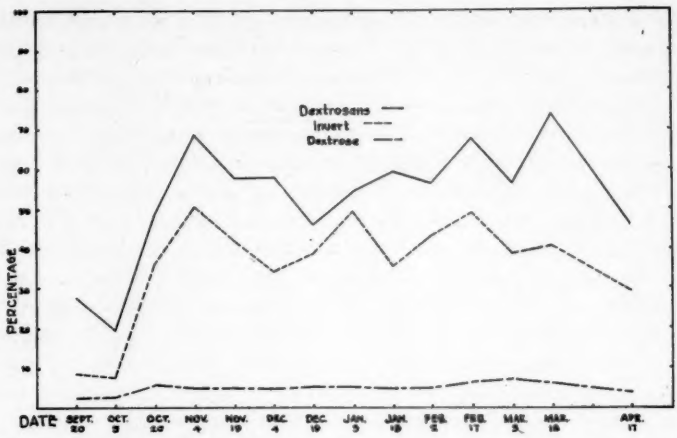

FIG. 4. Percentages of dextrose, invert sugar, and dextrosans occurring in *Pinus ponderosa* needles when based on dry weights.

plant. Catabolic processes, on the other hand, would tend to produce dextrose as a hydrolysis product of the reserves and as an intermediate stage in the plant's respiratory functions. Many have assumed that environmental factors do not affect variations in dextrose percentages directly, but only indirectly through their influence on photosynthesis and hydrolysis of reserves on the one hand and on respiration and the synthesis of reserves and structural materials on the other.

Figures 2 and 4 tend to accentuate the great variations in invert sugars throughout the colder seasons. No maximum was found in midwinter, as has been reported in numerous papers. This at first appears contradictory but, on a more detailed study, agrees in its entirety with the results of the above workers if based on temperature relationships.

It is, in general, to be noted that invert sugars undergo no periodic changes but necessitate the establishment of the nature and the rate of its metabolic rearrangements and disintegration as affected by the climatic conditions. CLEMENTS (**2**), working with the sunflower, and other investigators working with the apple and the maple, have reported actual migration of sucrose from the branches and petioles back into the leaves on the approach of cooler weather. This in itself would partially explain the relatively high constituent part of invert sugar in the October 20 sample, for both the pine and the fir. The high results of November 4 and thereafter are, however, probably due to the presence of certain decomposition products.

The gradual lessening of the starch content, and other polysaccharides, in other plants from September on, in opposition to the accelerated increases of the sucrose and the hexoses which are maintained throughout the winter, has been recorded in all research papers reviewed, save one. That a different state of affairs obtains in evergreen trees in regions of mild winter climate is indicated by CAMERON'S (1) investigation, which shows a constant increase in reserves during the autumn and winter. He obtained a maximum in early spring just prior to blossoming when the reserves constitute as much as 25 per cent. of the total fresh weight. MEYER (7), the only other worker on the carbohydrates in coniferous needles, did not measure the polysaccharides. The data represented by tables I and II or by figures 1 and 3 clearly reveal that the dextrosans did accumulate during autumn and did not decrease as recorded by most investigators. The higher peaks plotted require an explanation based upon interconversion into different types of compounds rather than upon actual storage of reserves. It is of interest to note that a definite relationship does not exist between the dextrosan and the invert sugar curves. For *Pinus ponderosa* from September 20 to October 5 and from December 4 to Jaunary 18, inclusive, and for *Pseudotsuga taxifolia* from December 4 to January 3, inclusive, the dextrosan curves increased and decreased concomitantly with the opposite accelerated change in the invert curves. At all other times a change in the one class was accompanied by a similar change in the other. There were no relative changes found in either case, and therefore the data do not authenticate a direct relationship between dextrosans and invert sugars. They do, nevertheless, indicate a possible relationship for the dates listed above, but even in these cases there is no correlation.

A marked modification of the starch content concomitant with changes in the temperature was observed by LIDFORSS (6), who showed that all European evergreen leaves in temperate latitudes are quite starch-free from the beginning of December throughout the winter. With the advent of higher temperatures in spring the starch again appears. MIYAKE (9) reported similar conditions existing in Japan. In the present investigation, the Soxhlet-residue was treated with iodine and examined microscopically. The results are tabulated in table III. Only once, on November 4 immediately following the sudden drop in temperature, was the Douglas fir void of starch. The data, taken as a whole, do agree with the results of the above papers; namely, the relative amount of starch decreases with a decrease in temperature and increases again with a return to higher temperatures. It is worthy of notice that with a change in relative amounts of starch there is often the opposite change in the quantity of dextrosans. This materially strengthens the hypothesis that the changes occuring in the dextrosan

TABLE III

RELATIVE AMOUNTS OF STARCH OBSERVED MICROSCOPICALLY*

DATE	*PSEUDOTSUGA TAXIFOLIA*	*PINUS PONDEROSA*
Sept. 20	Medium	Slight
Oct. 5	Slight	Medium
Oct. 20	Slight	None
Nov. 4	None	None
Nov. 19	Medium	Slight
Dec. 4	Medium	None
Dec. 19	Slight	None
Jan. 3	Slight	Slight
Jan. 18	Slight	None
Feb. 2	Slight	Slight
Feb. 17	Medium	Slight
Mar. 3	Medium	Much
Mar. 18	Much	Medium
Apr. 2	.....	.....
Apr. 17	Abundant	Abundant

* An arbitrary set of standards was employed.

extracts are due to changes in the types of reducing substances rather than actual changes in the quantity of reserves present.

An attempt was made to determine a possible correlation between saccharide fluctuations and climatic changes.

Meteorological data covering the entire period of this study were secured from the local Weather Bureau situated within an eighth of a mile of the arboretum where the trees were located.

Little doubt exists that certain metabolic functions within the plant are regulated by climatic changes indirectly. It is equally agreed that, of all the climatic factors, temperature is the most potent in its regulatory effect on the changes in carbohydrate quality. The influence of temperature on the carbohydrate content of plants such as conifers is, of course, the summation of the effect of this factor on a variety of activities; and, as a consequence, no mathematical correlation is to be expected. The data bear this out on the one hand, while on the other they establish certain relationships with the specific types of the elaborated foods. Since there are numerous ways by which temperature data can be represented, they were graphed in various forms to determine which most closely agreed with the carbohydrate data presented herewith. The curves were plotted from averages of the five days prior to each sampling. Whether the minimum temperature is the more important in its effect on certain enzyme actions causing the conversion of reserves into labile forms, or whether the maximum temperature in its regulatory rôle on respiratory and photosynthetic activities is the more important, is very difficult to ascertain.

A comparison of figure 5 with figures 1 and 3 obviates the necessity of detailed discussion and demonstrates the generalizations below. The dex-

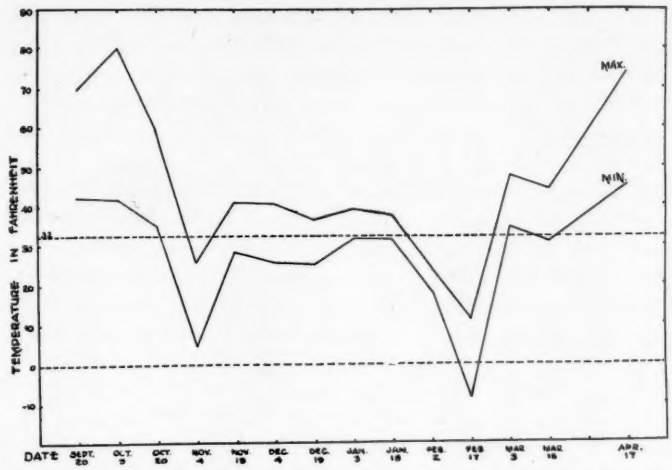

FIG. 5. Maximum and minimum temperature curves computed from averages of five days prior to each sampling.

trose content starts at a relative minimum and increases toward a maximum, which is limited to a small percentage of the total fresh weight, as the minimum temperature drops toward the freezing point. During the freezing temperatures the dextrose percentage remains comparatively near its maximum and fluctuates only slightly. This is strengthened by the results of JONES and BRADLEE (**5**), which tend to demonstrate that the so-called critical tree temperatures need not cover a very wide range above or below 32° F. within the tree and are governed by corresponding external air conditions.

The distinct gain in invert storage in the autumn months, which is shown with its maintenance, though in different proportions, throughout the winter months, does indicate a definite relationship with the prevailing temperatures. A superimposition of the invert sugar curves upon figure 5 definitely reveals an inverse relationship. This is universally accepted.

Except for CAMERON (**1**), all investigators have reported a decrease in percentage of reserve foods coincident with a lowering of the temperature and a redeposit of these carbohydrates with a return to the higher temperatures. Table III when compared with figure 5 shows that this relationship for starch holds for the coniferous needles concerned. The dextrosan curves, represented in figures 1 and 3, however, agree with similar measurements by CAMERON (**1**), on the orange. The sharp maxima in the curves can be explained only by decomposition products caused by the very sudden drops in the temperature during the days just prior to the taking of

the samples. A definite positive relationship between starch content and temperature appears to exist; while a negative relationship, just as striking in aspect, is evident between erratic temperature changes and the fluctuations in alcohol-insoluble acid-hydrolyzable reducing substances. When the temperature changes were gradual a positive relationship existed between temperature and this class of compounds.

Other habitat factors, although exerting less effect, might be considered. From data not included here, it was found that the range of available sunlight presented no measurable fluctuations. A similar comparison indicates that the water content may either diminish or accentuate the effect of the temperature. This depends whether a drop in the former is coincident with a drop in the latter or if a rise in the former and a lowering of the latter occur at the same time.

It was found by GAIL (3) that the osmotic pressure of expressed cell sap in some deciduous and non-deciduous plants reached a maximum during the winter months, and that there was a decrease in the spring and a gradual rise again with the arrival of autumn. He advocated the theory that this change of osmotic pressure was partially due to the changes in the sugar content. A plausible connection between variations in carbohydrates and osmotic pressure was sought; but, since no osmotic pressure measurements were made on the cell sap of the material involved, it was necessary to seek the relationship through an indirect procedure. It is possible, however, to compare GAIL's data and the data of this study by using the respective relationships with the temperatures as the medium. From such a comparison it is possible, and may be argued, as MEYER (7) has likewise pointed out, that the invert sugars in the needles serve to regulate the osmotic pressure owing to its ready interconversion to other forms. This regulation could be quite as well effected by the precipitation of the polysaccharides.

Summary

1. Quantitative measurements were made for three classes of carbohydrates in the needles of *Pinus ponderosa* and of *Pseudotsuga taxifolia* to determine their fluctuations during the more or less dormant seasons. The BERTRAND modification of the MUNSON and WALKER method was followed.

2. Reducing compounds other than sugars occurred in some of the extracts. Furfural and formaldehyde were definitely identified.

3. The hexose and pentose sugars, or better, the alcohol-soluble free reducing substances, maintained low percentages and limited variations during the colder seasons.

4. The distinct gain in alcohol-soluble acid-hydrolyzable reducing substances in the autumn months, with its maintenance, though in different proportions, throughout the winter months, indicates a definite inverse re-

lationship with the prevailing temperatures. Disaccharides and furfural are the major constituents of this group of compounds.

5. Alcohol-insoluble acid-hydrolyzable reducing substances, which contain starch, other dextrosans, and reserve hemicelluloses, increased rapidly immediately following a sudden drop in temperature; during gradual temperature changes a positive relationship existed.

6. The relative amount of starch decreased with a decrease in temperature and increased again with a return to the higher temperatures.

7. The changes occurring in the "dextrosan" extracts are due to a change in the type of reducing substances rather than an actual change in the quantity of reserves present.

8. The seasonal variations in the needles' soluble carbohydrate content, including hexoses, pentoses, and certain hemicelluloses, appear to be partially responsible for the seasonal variations in the osmotic pressure of the cell sap.

The writer wishes to express his gratitude to Dr. FLOYD W. GAIL for suggesting this problem and for his guidance throughout the investigation and preparation of the manuscript.

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SURVIVAL OF ISOLATED TOMATO ROOTS AT SUBOPTIMAL AND SUPRAOPTIMAL TEMPERATURES

PHILIP R. WHITE

(WITH ONE FIGURE)

In their early root-tip cultures, ROBBINS (**3, 4**) and ROBBINS and MANEVAL (**5, 6**) regularly made transfers to fresh media at about 2-week intervals. No reason is given by the authors for the choice of this period. KOTTE (**1**) and MALYSCHEV (**2**) used periods of various lengths, determined apparently by the increment rates of the individual cultures. In earlier work (**7, 8**), dealing with the effects of various external factors on growth of isolated roots, three components of the environmental complex: passage length, concentration of sugar, and concentration of yeast extract, were arbitrarily chosen to correspond with those used by ROBBINS. Since only the gross increments for the entire period were recorded, this passage length appeared to be satisfactory. In later work, however (**9**) when increments were recorded daily over an extended period of time instead of fortnightly, it became evident that the interval previously chosen was far too long for optimal results. The mean daily increments for 100 cultures measured daily for 25 weeks were, for the first 7 days: 3.6, 4.2, 5.6, 6.1, 5.9, 6.4, and 5.8 mm. per culture per day. The maximum rate of growth was thus reached somewhere between the 4th and 6th days. Thereafter the growth rates were not recorded regularly, but it was noted that they fell off gradually until, at about the 10th to 12th day, the increments were negligible. In most of the experiments subsequently carried out with these cultures, a period of one week, slightly longer than the optimum indicated by these figures, was employed, since such a period simplified the keeping of records.

A routine involving weekly transfers is satisfactory where, as is the case in most experimental work, a rapid turnover of cultures is desirable. However, when a great number of cultures has to be maintained, weekly transfers become costly of both materials and time. Such is the case, for example, with the group of stock cultures of virus-bearing roots maintained in this laboratory (**10**). A half-dozen different viruses, each in a separate set of roots, and each requiring a minimum of 10 replications for its proper maintenance, makes a total of 60 cultures. It is anticipated that ultimately many more viruses will be maintained in root cultures.

Although optimal growth did not ordinarily occur at room temperature after the 6th day, cultures were in some cases kept alive over very much greater periods of time without transfer. Roots left for long periods with-

out transferring ceased to grow normally; the growing points became swollen and crooked, and often turned brown. After a period of dormancy, new branch roots were put out, often in clusters just back of the old growing points, and active growth was resumed for a time. These new rootlets finally stopped growing, the same symptoms of injury appeared, and a second period of dormancy supervened, followed by the formation of still another set of new growing points. Such a cycle might be repeated several times, the cultures remaining alive even after the culture solution had evaporated down to a small fraction of its original volume. Cultures of clone B (**9**) were recovered on one occasion after being allowed to stand without transfer for 145 days. Cultures so treated, however, were subjected to considerable danger of ultimately becoming contaminated by penetration of molds through the cotton plugs, and, although roots so infected were frequently recovered in an aseptic condition, the risk of loss was great. Moreover, when a root was allowed to grow undisturbed for a long period, even though the growth rate was definitely suboptimal, the flask became filled with a tangled mass of branching rootlets from which it was difficult to isolate and remove satisfactory fragments for subculture. The handling involved in the isolation of subcultures from such masses greatly increased the danger of loss by contamination. Furthermore, recovery of such material upon transfer to fresh nutrient was in a considerable percentage of cases slow and irregular. This procedure was obviously not to be relied upon as a routine method of maintaining cultures for long periods.

With these observations in mind, it seemed worthwhile to attempt to develop procedures by which the length of time between transfers could be increased without seriously impairing the vitality of the roots. The temperature-growth-rate curve for isolated tomato roots has previously been shown (**11**) to be extremely sharp, with a maximum at 30° C. Since growth was reduced both above and below this point, the use of suboptimal and supraoptimal temperatures seemed to offer a possible means of increasing the passage length. The present paper describes experiments planned to deal in greater detail with the effects of such temperatures.

Experimentation

As was shown elsewhere (**11**), growth of isolated tomato roots was practically *nil* at 5°, 8°, 10°, and 40° C., and took place at a very slow rate at 15° and 35° C. Growth at temperatures between 20° and 33° C. was fairly rapid and could be maintained indefinitely, but at 32° and 33° C. the roots were often discolored and evidently in poor condition. To determine whether the submaximal growth rates obtained at suboptimal and supraoptimal temperatures were due to injury or merely to temporary retardation of the metabolic processes, cultures kept for one week at each of the

temperatures mentioned above, with the exception of 32° and 33° C. were subsequently transferred to room temperature (*ca.* 22° C.) for a second week. Growth rates were recorded for both passages. In all cases, with the exception of those kept at 35° and 40° C., the growth rates at 22° C. were normal, that is, recovery was complete (fig. 1). Cultures grown at 35° and 40° C. and then transferred to 22° C., on the other hand, made no recovery.

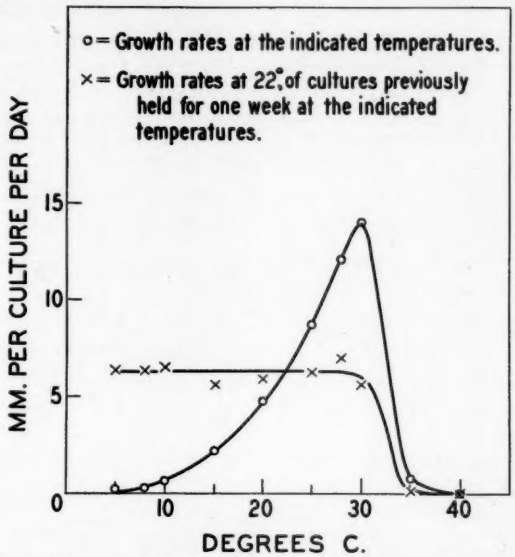

FIG. 1. Growth rates of isolated tomato roots grown for one week at a series of controlled temperatures and then transferred for a second week to a temperature of about 22° C.

It was evident that, while brief exposure to suboptimal temperatures not reaching the freezing point was not seriously detrimental to isolated roots, similar exposure to supraoptimal temperatures did seriously injure them. Since it is known (**11**) that continuous growth at a moderate level can be maintained at 20° C. (winter room temperature), further study was restricted to temperatures below this level. Growth was studied in detail at two temperatures, about 8° and 15° C.

Survival at about 8° C. was tested over periods of from 1 to 10 weeks (March 5 to May 14, 1934). One hundred cultures from clone C (**9**) were prepared simultaneously and measured. They were placed together in a darkened cellar room having at the beginning of the experiment a temperature of 6.7° C. and at the end of the 10-weeks' period a temperature of 16° C. The highest temperature reached while viable roots remained in the

room (see below) was 11° C. The gradual rise in the temperature was due to the coming of spring. Ten cultures were removed at the end of each week to a laboratory having a temperature of about 22° C. and were measured. They were allowed to grow and were transferred each week. This was continued until either they had resumed a rate of growth equal to that in the controls, or else had shown clearly that they would not attain such a growth rate within a period suitable for routine use. The approximate growth rates of this series expressed as percentages of the controls are shown in table I. After the first week growth at 8° C. was too little to be measured.

TABLE I

INCREMENT RATES OF ISOLATED TOMATO ROOTS GROWN FOR VARIOUS PERIODS AT 7–16° C. AND THEN TRANSFERRED TO 22° C.

		TEMPERATURE	WEEKS→ 1	2	3	4	5	6	7	8	9	10
	0	7	100	100	100	100	100	100	100	100	100	100
Weeks	1	7	5	121						Growth rates		
	2	8	5	0	40	148				at 22°		
before	3	8	5		0	0	0	120				
	4	10	5			0	0	95	140			
transfer	5	11	5				0	0	6	110		
	6	11	5			Growth rates		0	0	0	0	0
to	7	12	5			at 7–16°			0	*	–	–
	8	13	5							*	–	–
22°	9	15	5							*	–	–
	10	16	5							*	–	–

* Contaminated with molds.

Recovery of each group as a whole was complete after 1, 2, 3, 4, and 5 weeks. At the end of this time the temperature of the culture room was 11° C. Recovery did not occur when cultures were exposed for longer periods. After one week, 9 of the 10 cultures resumed growth from the old growing points. The same was true after 2 weeks. But after 3 weeks all original growing points were dead and the subclone was recovered from newly laid down lateral initials in 3 cultures which survived out of the original 10. After 4 weeks, 4 cultures survived and produced viable laterals, but were without viable terminal growing points. After 5 weeks, 3 cultures survived in the same way. After 6 weeks, 2 cultures survived but grew very poorly. Seven weeks after beginning the experiment, all cultures in the experimental room became simultaneously contaminated with molds. This general contamination was probably due to the excessively moist conditions prevailing in the room at this time, which allowed molds to develop on and penetrate the cotton plugs protecting the flasks.

It is evident from the data presented above that, although recovery *may* occur after 5 or 6 weeks at 7–11° C., such recovery is uncertain after more than 2 weeks. Temperatures of 10° C. or less thus offer little if any advantage over room temperature for the maintenance of roots.

Survival at 15° C. was tested in the same way, using a constant temperature oven controllable to ± 2.0°. Eighty cultures were prepared and 10 cultures removed after each week for 8 weeks. The results are shown in table II. The mean growth rate for all cultures at 15° C. was 1.6 mm. per

TABLE II

INCREMENT RATES OF ISOLATED TOMATO ROOTS GROWN FOR VARIOUS PERIODS AT 15° C. AND THEN TRANSFERRED TO 22° C.

	WEEKS→	1	2	3	4	5	6	7	8	9	10
	0	100	100	100	100	100	100	100	100	100	100
Weeks	1	30	164	147						Growth rates	
at	2		30	106	147					at 22°	
15° C.	3			30	83	140					
before	4				30	43	120				
transfer	5		Growth rates			30	80	163			
to	6		at 15°				30	146	156		
22°	7							30	150	159	
	8								30	123	197

culture per day, about 30 per cent. of that of the control. At every period studied the roots showed some recovery in the first week after exposure to room temperature, and in only three cases (3, 4, and 5 weeks) were the experimental cultures exceeded by the controls. When held for 2 weeks at room temperature, all experimental groups recovered completely, and at the end of this time all were growing *more* rapidly than the controls. The mean percentage index for all groups was 153, and the lowest index, that for 4 weeks at 15° C., was 121. It is thus evident that such roots could not only be kept without transfer for at least 8 weeks at 15° C. without injury, but that they were actually stimulated by such treatment.

These figures, however, do not tell the whole story, for the original growing points of all cultures kept for more than 5 weeks at 15° C. were killed, recovery in these cases being from newly formed laterals. Symptoms of injury similar to those occurring at room temperature after 10 to 12 days appeared in these cultures after 4 to 5 weeks.

It seems probable from these results that the temperature at which truly satisfactory growth can be maintained with the greatest length of passage should lie somewhere between 8° C., at which no growth occurred, and 15° C., at which the growth rate was reduced to about 30 per cent. of its value at room temperature. For practical purposes, however, a more exact analysis is unnecessary. By keeping cultures at 15° C., isolated roots can be

maintained in good condition without transferring oftener than once in 2 months. The application of such a method in the maintenance of stock cultures is evident.

Summary

Isolated tomato roots kept at 7–11° C. for longer than two or three weeks without transfer die. If kept at 22° C. they must be transferred weekly if they are to be maintained in good condition. At 15° C. they will remain in good condition if transferred at intervals of two months. A temperature of about 15° C. is thus more suitable than 8° C. or 22° C. for the maintenance of large numbers of stock cultures.

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SEPARATION FROM YEAST OF MATERIALS ESSENTIAL FOR GROWTH OF EXCISED TOMATO ROOTS

PHILIP R. WHITE

(WITH ELEVEN FIGURES)

Introduction

Three years ago the results of experiments on the cultivation of excised tomato root-tips, *in vitro*, for a period of one year were reported (**7**). From the data available at that time it was concluded that such roots could be kept growing and be maintained in apparently normal condition for potentially unlimited periods of time. The environmental complex used was shown to be adequate for all requirements of growth. Aside from possible traces of impurities in the salts and sugar used, only one factor of the environmental complex, namely, the extract of yeast, amounting to 0.01 per cent. of the nutrient, was recognized as unknown. Identification of the materials in this unknown and their replacement by known materials were considered important problems for the future. The idea that any growth-limiting material, such as a hormone, might be furnished to the culture from the parent plant was shown to be untenable. Since, however, such diverse materials as vitamines, auxins, auximones, bioses, and nutrilites, as well as biologically active sugars, accessory mineral elements, haemin, sex hormones, insulin, nucleic acids, porphyrins, and many other materials of uncertain physiological significance have been recorded as constituents of yeast, the presence of the yeast extract in the nutrient obviously represented a serious stumbling block to any definite conclusions as to the nutritional requirements of the plant tissues under consideration. The fact that ROBBINS (**2**) using a nutrient-containing peptone, WHITE (**4**) using a fibrin digest, KOTTE (**1**) and WHITE (**4**) using meat extract (LIEBIG), and KOTTE (**1**) using various amino acids, all obtained some growth, suggested that the constituents necessary for normal growth might not be specific to yeast, but none of them had been identified. The work presented here was planned to throw light on the nature of these constituents.

Materials and methods

Roots for study were of a single clone obtained from the tomato variety Bonny Best. They had been kept in continuous culture for more than two years (**8**). All tips used were selected carefully for uniformity in appearance. Successive series of 14 to 16 different nutrient solutions, each represented by 20 cultures, were grown under as uniform conditions as were obtainable in an ordinary diffusely lighted laboratory. Each series, unless

otherwise, stated, included two sets of controls one employing the standard nutrient developed in previous work (**5, 6**), the other the same nutrient with the yeast extract omitted. The results obtained in the 12 to 14 nutrients under investigation in each series were then compared with those given by one or both of the controls.

The method of preparing the standard nutrient (**6, 7**) has been somewhat modified. The constituents were made up in five solutions containing: (1) nutrient salts, (2) "accessory" salts, (3) iron, (4) sugar, and (5) accessory organic materials. The nutrient salt solution was prepared as follows: 140 gm. $(CaNO_3)_2 \cdot 5H_2O$, 67 gm. KCl, 81 gm. KNO_3, and 12.25 gm. KH_2PO_4 were dissolved in 8 liters of distilled water. 74 gm. $MgSO_4 \cdot 7H_2O$ were dissolved in 2 liters of water. The second solution was then poured slowly into the first with constant stirring. A slight precipitate eventually formed but was not carried over into the final nutrient. Ten ml. of this stock solution were sufficient for a liter of nutrient. The "accessory salts" solution contained 300 mg. $ZnSO_4$, 270 mg. Na_2SiO_3, 1070 mg. $Al_2(SO_4)_3$, 150 mg. KI, 320 mg. H_3BO_3, 560 mg. NaCl, 440 mg. $MnSO_4$, 40 mg. $NiCl_2$, 39 mg. LiCl, 40 mg. $CoCl_2$, and 10 mg. $CuSO_4$ in ten liters of water. As in the case of the nutrient salt solution, 10 ml. of this solution were sufficient for 1 liter of nutrient. The iron and sugar were made up fresh each time nutrient was prepared; 2.5 mg. $Fe_2(SO_4)_3$, and 20 mg. sucrose were added to each liter of nutrient. The accessory organic materials were the only variables in the experiments to be reported here. In the nutrient used for cultures designated as "controls without yeast," such materials were entirely omitted. The "controls with yeast" contained yeast material prepared as follows: 25 mg. of yeast ("Brewer's Yeast—Harris") were boiled for ½-hour in 2 liters of distilled water. While still hot the preparation was centrifuged for 5–10 minutes at high speed. The supernatant fluid was decanted, made up to 2.5 liters and distributed to Pyrex test-tubes in 50-ml. aliquots. These were autoclaved at one atmosphere pressure for 20 minutes, cooled, and stored for future use at – 15° C. Ten ml. of this preparation were sufficient for 1 liter of nutrient. One liter of the standard control nutrient then contained 10 ml. of the nutrient salt solution, 10 ml. of the accessory salt solution, 10 ml. of the yeast preparation, 2.5 mg. $Fe_2(SO_4)_3$, and 20 gm. sucrose.

The roots were measured daily, and the growth increments of the 20 (sometimes 10) cultures in each nutrient averaged. Most series were repeated two or more times. The results have been plotted as average increments in length expressed in terms of millimeters per culture per day. The curves obtained indicate the increment trends clearly. The experiments reported were made during the period from September, 1934, to May, 1936.

Experimental results

Yeast extract was shown by ROBBINS (**2**) and WHITE (**5**) to be beneficial to isolated root-tips. ROBBINS found that the stimulative effect did not become evident until after several passages (**2**). WHITE's experiments with yeast were carried out for a single passage only and were, therefore, unsuitable for determining any but the gross requirements of the tissue. Moreover, the measurements recorded by both authors were made only at the *end* of one or more passages of 2 weeks' duration. It seemed possible that the growth recorded might all have occurred in the first part of the passage, at the expense of material from the tissue of the initial explant. Thus, neither author demonstrated unequivocally the essential nature of these yeast materials. To determine whether or not records of daily growth rates would throw light on this problem, 10 cultures were made in a nutrient lacking yeast material, with 10 controls in a complete nutrient. The increments were recorded daily for one week. The results are plotted in figure 1. The average *total* increment in the first passage, in the absence of yeast, was 41 per cent. of that obtained in the control nutrient, a figure comparable to that previously recorded by ROBBINS (**2**) and by WHITE (**7**); but the average *final* daily increment rate in the absence of yeast was only 13 per cent. of the control, indicating that very little growth was possible as the end of the period was approached. Subcultures were made from each set to a nutrient similar to that used during the first week. The growth rate of the experimental cultures decreased still further, so that at the end of the second passage the increments at the original growing points in the experimental series were *nil* and the growth that occurred in the most rapidly growing branches was only about 2 per cent. of that recorded for the controls. Addition of yeast ash (**3**) on the 5th day of the second passage did not increase growth.

The slow rate of increment at the end of the second passage is not, however, indicative of lack of vitality in the cultures. More branches were formed in the absence of yeast than in its presence. But these branches, although in apparently healthy condition, grew for only a short time. The tips soon died, and new branches were formed. This suggested that essential nutrients, freed by the dying of the older growing points, were reutilized in forming new ones. When cultures grown in a deficient nutrient were transferred at the end of the second passage to a complete nutrient, the increment rates rose sharply on successive days from 0 per cent. to 46, 91, 110, 140, and 180 per cent. of the controls. In the fourth passage, this high level was at first maintained but fell off gradually until in the fifth passage both groups were growing at approximately the same rate. Similar behavior was observed when roots were subjected to sub-optimal temperatures (7–10° C.) for a time and then returned to room temperature (**9**). This observation seems to indicate that adverse conditions may slow down certain meta-

bolic processes while leaving others unaffected, permitting storage of excess quantities of some material which under normal growing conditions is a

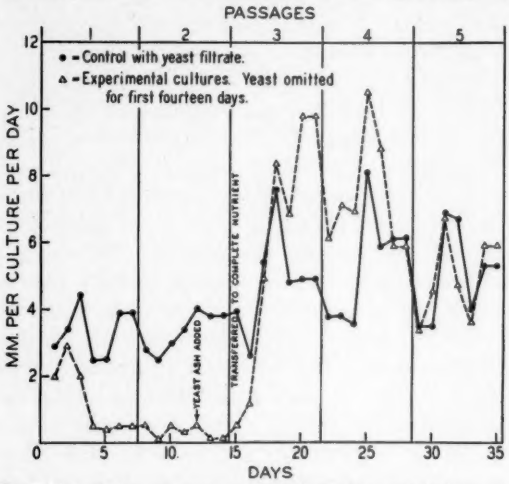


FIG. 1. Effect of omission of yeast extract from the nutrient for 2 passages,* followed by return to a complete nutrient for 3 passages thereafter.

Photograph by J. A. CARLILE

FIG. 2. Upper row, roots grown for 3 weeks in nutrient lacking yeast extract. Lower row, roots grown without yeast extract for 2 weeks, then returned to a complete nutrient for 1 week. ×0.5.

* "Passage 1" in this experiment is passage 76, counting from the time when the clone was isolated. The roots had been maintained through 75 passages under standard conditions before being used in this experiment. Other experiments recorded here were carried out with roots previously maintained for from 75 to 160 passages under standard conditions.

limiting factor. In further experiments roots kept in a nutrient lacking yeast for periods of 1, 2, 3, 4, and 5 weeks all recovered when transferred to a complete nutrient. Examples of such roots are shown in figure 2.

The results indicate clearly that some material supplied by yeast extract *is* essential for satisfactory growth. The rapidity with which this deficiency shows up, as indicated by the abrupt divergence between the curves of figure 1, shows that this material is carried over with the explant in smaller amounts than are required to sustain normal growth over a period of one week or longer. These results have been verified repeatedly in the controls of a number of different experiments. It is evident that under the experimental conditions used here, the form of the growth curve within a single passage can be taken, tentatively at least, as an indicator of the efficiency of any nutrient.

A standard concentration of an extract of 100 mg. dried Brewer's yeast (Harris) per liter of nutrient was used in all experiments up to this point. The choice of concentration to be used was based on the work of ROBBINS

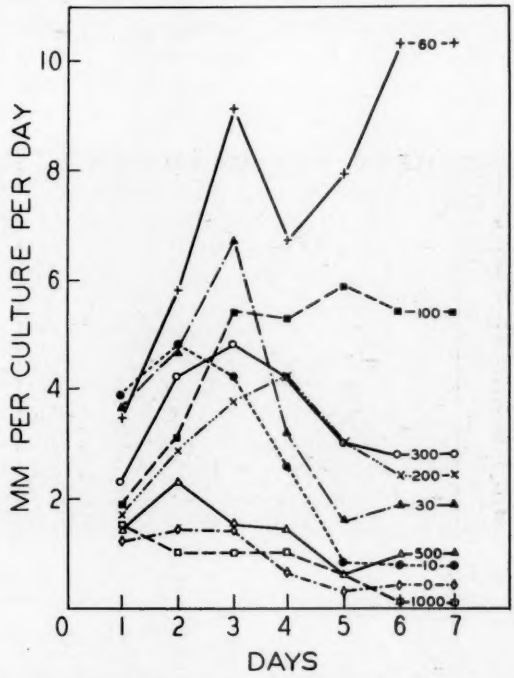


FIG. 3. Curves showing growth rates in nutrients containing extracts of from 10 to 1000 mg. of yeast per liter.

(**2**), and was about the minimum which he found satisfactory. Since the yeast employed by ROBBINS was autolyzed, while the preparation used in this work was pasteurized at the height of its activity so that only the free, water-soluble materials were available, it seemed desirable to determine the concentration of these materials that would prove optimal for growth. Nutrient solutions containing 10, 30, 60, 100, 200, 300, 500, and 1000 mg. of yeast per liter were therefore tested. The results are shown in figure 3. They indicate that the standard concentration of 100 mg. of yeast per liter, employed in the past, was close to the optimum, being exceeded by only a single concentration, 50 mg. per liter. This concentration has, therefore, been retained for all subsequent work.

Since yeast does evidently furnish some material or materials essential for growth of tomato root tips, and the optimal concentration of the raw extract was known, an attempt was made to isolate this material or materials by various types of fractionation.

SOLUBILITY IN WATER

To determine whether or not the growth-promoting material was completely extracted by boiling water, 50 gm. of yeast were boiled for ½ hour in

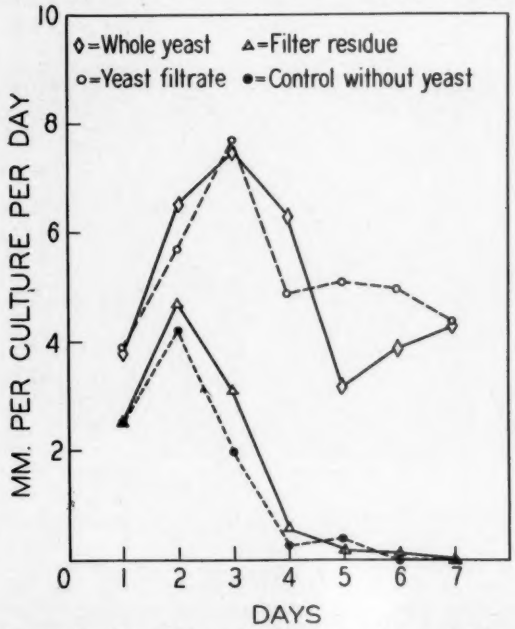


FIG. 4. Curves showing effects of fractions of yeast separated by filtration.

1 liter of water, filtered, the residue re-extracted in like manner twice more, the extracts mixed, and equivalent aliquots of extract, of residue, and of whole yeast resuspended in nutrient and tested. The results are shown in figure 4, and indicate that water extraction does remove all of the effective material. Like results were obtained when centrifugation was substituted for filtration. Aliquots dried *in vacuo* and weighed showed that the water-soluble material made up 36 per cent. of the total dry weight of the yeast. The residual 64 per cent. was inert as regards growth-promoting properties.

Diffusibility

A water extract of yeast was subjected to dialysis through a cellophane membrane. A stirring device was used to insure thorough mixing. The extract was placed within a tubular membrane which was immersed in a quantity of water approximately equal to that within the membrane. The external water was renewed every hour for 6 consecutive periods, the 6 extracts being finally mixed. Assuming complete equilibrium between external and internal solution at the end of each period, the amount of dialyzable material left within the membrane at the end of this treatment should be about 1.5 per cent. of that originally present, 98.5 per cent. having passed into the diffusate. The growth-promoting properties of the two fractions are shown by the data presented in figures 5 and 6. Most of the effective material passed through the membrane. The small residual activity of the non-diffusible fraction was probably due to incomplete dialysis. The diffusible material represented 23 per cent. of the weight of whole yeast. At least 77 per cent. of the yeast was thus shown to be inert.

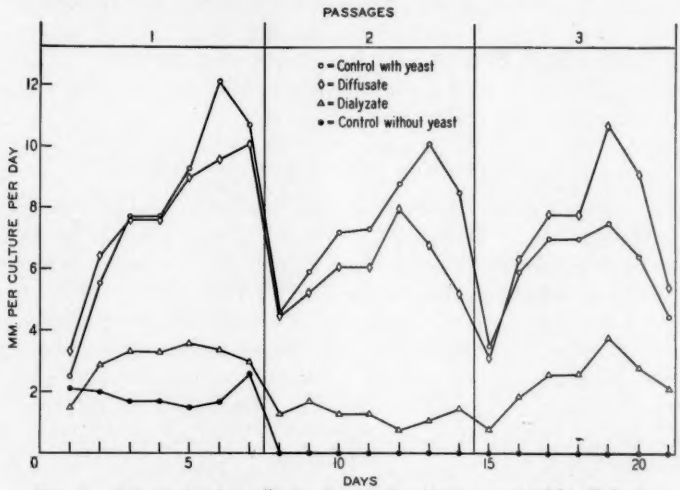
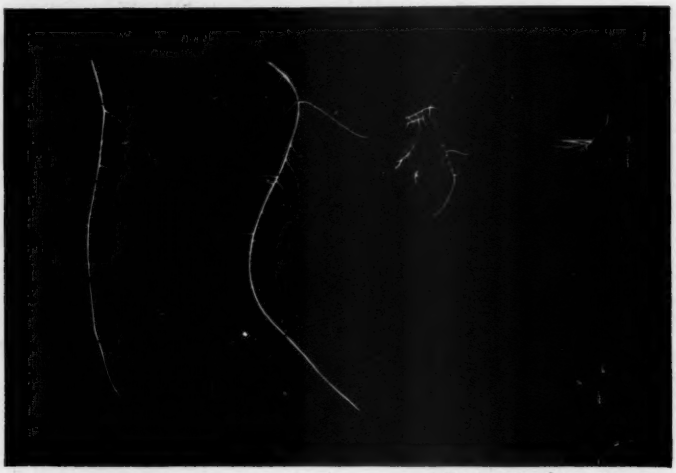


Fig. 5. Curves showing effects of yeast fractions separated by dialysis.



Photograph by J. A. CARLILE

FIG. 6. Effects of fractions of yeast separated by dialysis. Reading from left to right: A, control with whole yeast extract. B, fraction passing through cellophane. C, fraction retained by cellophane. D, control without accessory materials. Photograph taken at the end of the 3rd passage. × 0.6.

SOLUBILITY IN 85 PER CENT. ALCOHOL

An aliquot of a water extract of yeast was reduced to dryness over a steam bath and then extracted 3 times with boiling 85 per cent. ethyl alcohol, refluxing for ½ hour with each extraction. The extracts were mixed, both fractions dried, the dry residues resuspended in water and tested. The results are shown in figure 7, and demonstrate that all of the growth-promoting material was soluble in 85 per cent. alcohol. This fraction represents 18 per cent. of the total weight of dry yeast.

SOLUBILITY IN 100 PER CENT. ALCOHOL

An 85 per cent. alcohol extract of yeast was dried and then extracted 3 times with portions of absolute ethyl alcohol freshly distilled over CaO to insure complete dryness. The fractions were dried, redissolved in water and tested, both separately and in combination. A small portion of the 100 per cent. alcohol-soluble material prepared in this way failed to redissolve. This insoluble material was dark brown, highly aromatic, and oily. It proved to be soluble in ether, slightly soluble in alcohol, and insoluble in water. It appears to be lipoidal in nature, and was presumably carried over in an emulsified form in the previous extraction. The fractions soluble in water but separated by extraction with absolute alcohol were tested separately and

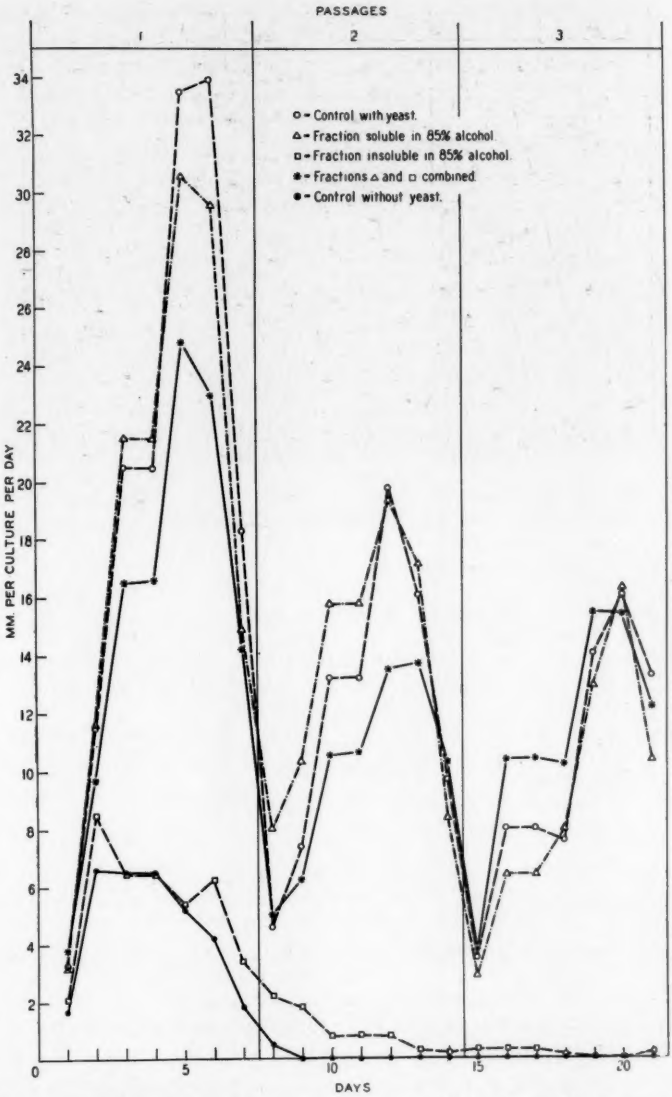


FIG. 7. Effect of yeast fractions separated on the basis of solubility in 85 per cent. alcohol. The differences between growth rates in different passages are the result of uncontrolled temperature and are to be ignored. The growth rate in the 1st passage was extraordinarily high, the 2nd and 3rd passages being more nearly average.

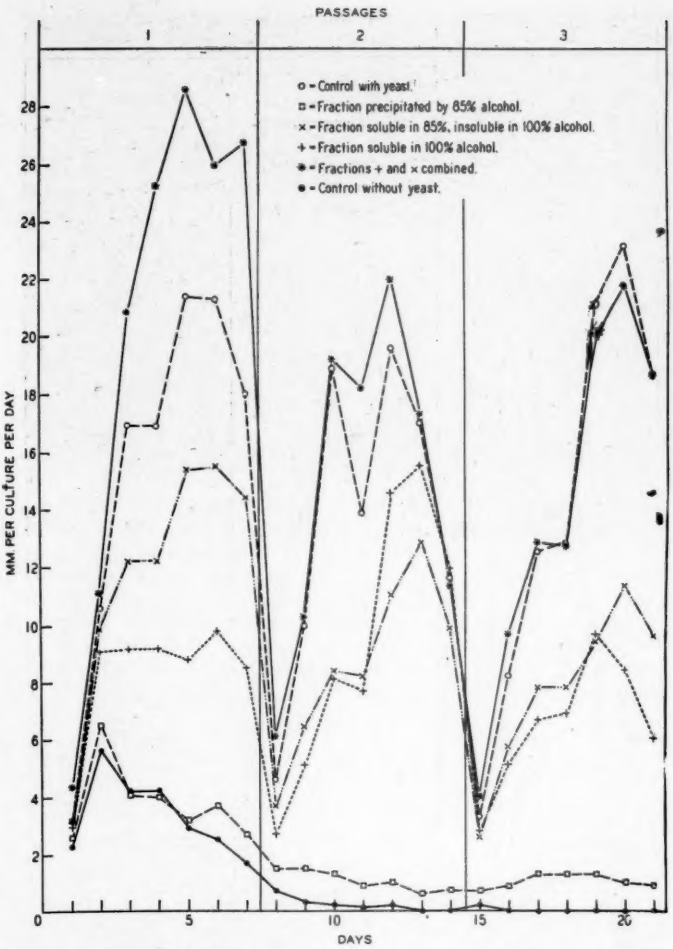
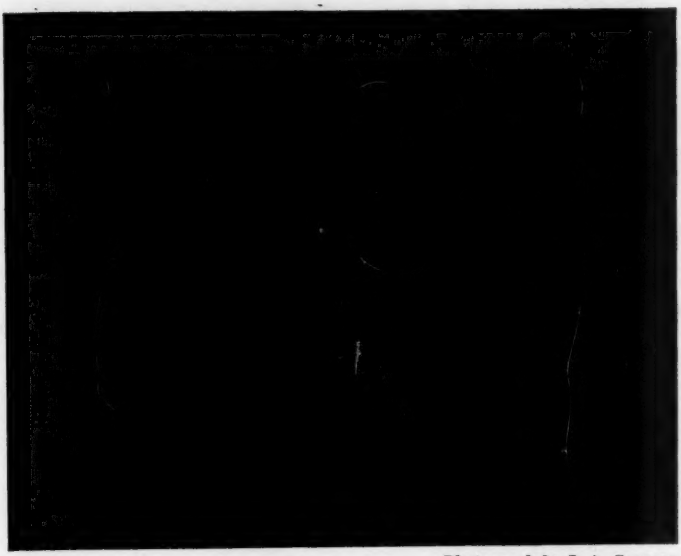


FIG. 8. Effects of yeast fractions separated by extraction with 85 and 100 per cent. alcohol.

in combination. As will be seen from figures 8 and 9, this treatment separates the yeast material into two portions, either of which will maintain growth continuously at a low level. Both are necessary for satisfactory growth. This is the first indication obtained that more than one accessory substance or group of substances obtainable from yeast are required by tomato roots. The material soluble in 85 per cent. alcohol but insoluble in



Photograph by J. A. CARLILE

FIG. 9. Effects of yeast fractions separated on the basis of solubility in 85 and 100 per cent. alcohol. Reading from left to right: A, control in whole yeast extract. B, control without accessory material. C, material precipitated by 85 per cent. alcohol. D, material soluble in 85 per cent. alcohol, insoluble in 100 per cent. alcohol. E, material soluble in 100 per cent. alcohol. F, fractions D and E mixed. Photographs taken at the end of 3 passages. × 0.5.

100 per cent. alcohol represented 13 per cent. of the total weight of yeast, that soluble in 100 per cent. alcohol, 5 per cent.

SOLUBILITY IN ETHER

An 85 per cent. alcohol extract of yeast was dried and then extracted 3 times with ethyl ether. Figure 10 shows the results of testing the fractions separated in this manner. The effective material was insoluble in ether and therefore not lipoidal. The ether-soluble fraction prepared in this way represented about 0.7 per cent. of the weight of dry yeast.

STABILITY IN TRICHLOROACETIC ACID

An 85 per cent. alcohol extract of yeast was dried, a 40 per cent. solution of trichloroacetic acid added, the mixture boiled until the odors of acetic acid and of chlorine were no longer detectable, and then cooled and adjusted to the original pH (about 5.5). No precipitate occurred during this treatment, and on testing, practically all of the activity was recoverable in the material so treated (fig. 11). The active material is, therefore, probably not

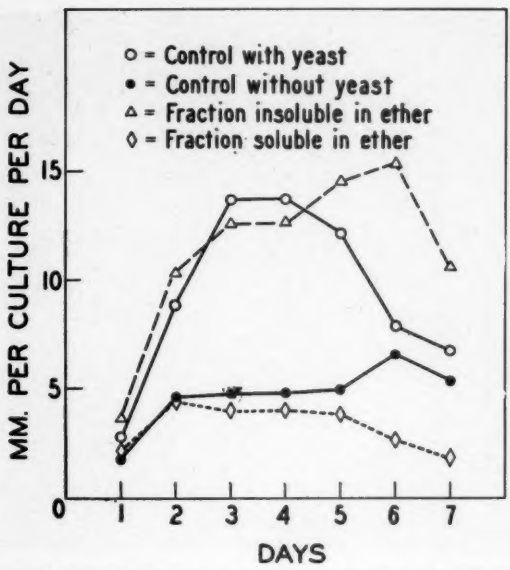


FIG. 10. Effect of yeast fractions separated on the basis of ether solubilities.

proteinaceous in nature, but such treatment does not eliminate the possibility of its being proteose.

TABLE I

SUMMARY OF RESULTS

TREATMENT	WEIGHT AS PERCENTAGE OF WEIGHT OF WHOLE YEAST		GROWTH-PROMOTING EFFECT OF MATERIAL TREATED
	INSOLUBLE	SOLUBLE	
Water	64	–	–
	–	36	+++
85 per cent. alcohol	16	–	–
	(2)*	18*	+++
100 per cent. alcohol	13	–	+
	–	5	+
Ether	4.6	–	+++
	–	0.4	–
Trichloroacetic acid	–	–	+++
Nitrous oxide	–	–	–

* About 2 per cent. of this fraction was lost in the process of preparation due to volatilization and other unavoidable causes. This has been placed in the "insoluble" column so as to account for all the weight.

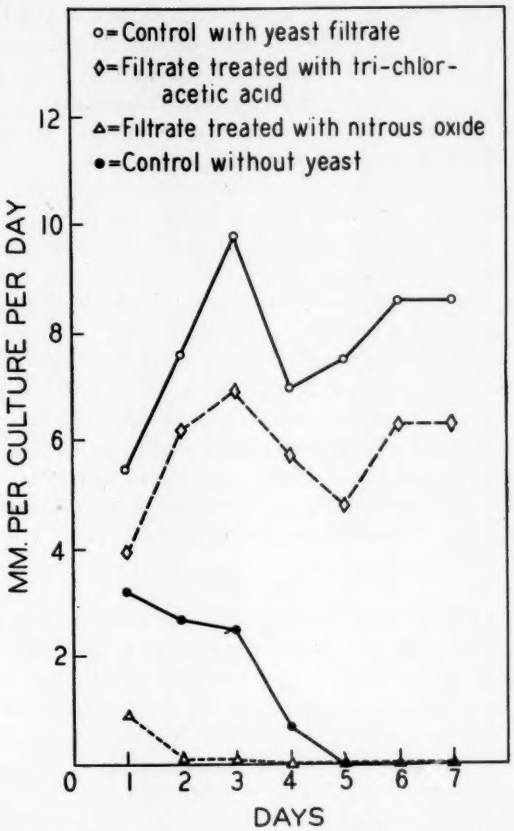


FIG. 11. Effects of treatment of yeast extract with boiling trichloroacetic acid and with nitrous oxide.

STABILITY IN NITROUS OXIDE

An aliquot of yeast extract was shaken for ½ hour in an atmosphere of NO_2 at pH 3, the pH readjusted to 5.5, and the growth-promoting properties tested. As shown in figure 11, this treatment completely destroyed the effectiveness of the extract. This suggested the probable importance of amino-groups.

The results of this analysis are summarized in table I.

Conclusions

From the results of this preliminary fractionation it is clear that, since the yeast material which exerts an essential growth-promoting effect on iso-

lated tomato roots is insoluble in ether, it cannot be lipoidal in nature, nor can it be one of the generally recognized growth "hormones" such as "auxin" or "hetero-auxin." It is equally clear that, being soluble in 85 per cent. alcohol, stable in trichloroacetic acid, and stable to repeated boiling, it cannot be any protein unless possibly prolamine. On the other hand, its destruction by nitrous oxide suggests that the amino groupings may play an important rôle in bringing about its growth-promoting effects. The material soluble in absolute alcohol, which is essential for satisfactory growth, cannot contain amino-acids other than small amounts of prolines, but the other effective fraction, insoluble in 100 per cent. but soluble in 85 per cent. alcohol, must contain considerable amounts of amino acids. A tentative analysis has shown amino acids to represent 20 to 30 per cent. of this fraction. They constitute a possible source of the growth-promoting properties of the absolute alcohol-insoluble fraction. Since this fraction makes up only 13 per cent. of the weight of whole yeast and is itself about 25 per cent. amino acids, effective amino acid materials cannot, in the yeast extract, exceed a concentration of about 4 mg. per liter of nutrient. The fraction soluble in absolute alcohol is likewise effective at a concentration of 4–5 mg. per liter of nutrient. Further analysis will be discussed elsewhere.

Summary

Material obtainable from yeast has been shown to be essential for satisfactory growth *in vitro* of excised tomato roots. Optimal results were obtained with about 100 mg. of yeast per liter of nutrient. Treatment of yeast with various reagents showed all of the effective material to be soluble in H_2O and 85 per cent. ethyl alcohol, stable in trichloroacetic acid, unstable in nitrous oxide, and insoluble in ethyl ether. At least 82 per cent. of the yeast material is inert, only 18 mg. of an 85 per cent. alcohol extract per liter of nutrient being needed for optimal results. Extraction of this material with 100 per cent. alcohol separates it into two fractions, both of which are essential for satisfactory growth. The material insoluble in 100 per cent. alcohol contains considerable quantities of amino acids, and it is suggested that these may play an important rôle as growth-promoting substances for isolated tomato roots.

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AMINO ACIDS IN THE NUTRITION OF EXCISED TOMATO ROOTS

PHILIP R. WHITE

(WITH FIVE FIGURES)

Introduction

A preliminary study of the growth-promoting materials obtainable from yeast and essential for the nutrition of excised tomato roots (**10**) has shown that all of these materials are soluble in 85 per cent. ethyl alcohol, insoluble in ether, and stable in boiling trichloroacetic acid. They can be further divided into two fractions by extracting with absolute alcohol. Both fractions are essential for satisfactory growth. The present paper deals with results of an analysis of that fraction which is soluble in 85 per cent. alcohol and insoluble in 100 per cent. alcohol and ether. The basic nutrients, cultural methods, and materials used have been the same as those previously described.

As was stated in the earlier report, the material soluble in 85 per cent. alcohol contains, in addition to many unknown substances, a very considerable amount of free amino acid. OSBORNE and WAKEMAN (**7**) give the total N content of a similarly prepared fraction, soluble in 80 per cent. alcohol, as 6.5 per cent. of the total yeast N or 12 per cent. of the weight of the fraction. They do not report the amino nitrogen content. About 3.5 per cent. of the total weight of this fraction, or 29 per cent. of its total N, has been found by the present writer to be amino nitrogen (van Slyke method). Between 20 per cent. and 25 per cent. of the fraction is, therefore, amino acid. As was suggested in the earlier paper, this high amino acid content obviously suggests that this constituent may play an important part in bringing about the growth-promoting activity of the fraction. As a check on this possibility an aqueous extract of yeast was shaken for one-half hour in an atmosphere of NO_2 at pH 3.0, adjusted to pH 5.6, and its growth-promoting properties tested. The effectiveness was found to be completely destroyed. One of the primary effects of this treatment is deaminization, although such treatment is too drastic to furnish an unequivocal diagnosis. This observation supports the presumption that the amino acids might be important factors in producing the effectiveness of the fraction. It seemed desirable, therefore, to determine in some detail the effects of commonly occurring amino acids on growth of these roots.

Experimentation

EFFECTS OF KNOWN AMINO ACIDS ON GROWTH

In a preliminary survey, 19 nutrients were made up, each one containing ing the usual salts and carbohydrate but with a single amino acid taking the

place of accessory organic material. Amino acids were obtained from two sources, as follows: From Dr. C. S. MARVEL, University of Illinois, l-cystine, l-tyrosine, dl-valine, dl-isoleucine, dl-norleucine, dl-methionine, dl-phenylalanine, dl-lysine-2HCl. From Pfanstiehl Chemical Co., Waukegan, Illinois, glycine, d-arginine, l-leucine, l-tryptophane, l-proline, l-hydroxyproline, l-histidine-HCl, l-aspartic acid, d-glutamic acid, dl-serine, dl-alanine. Hydroxy-glutamic acid, citrulline, and ROSE's α-amino-β-hydroxy-n-butyric acid (1) were not available for study.

A concentration of 5 mg. of amino acid per liter of nutrient (5 p.p.m.) was first tested. This was slightly less than the total weight of amino acids (8 mg.) shown by MEISENHEIMER to be present (2) in what has been determined to be the optimal concentration of yeast (10). The results are shown in figure 1. At a concentration of 5 mg. per liter, 7 of the 19 amino acids

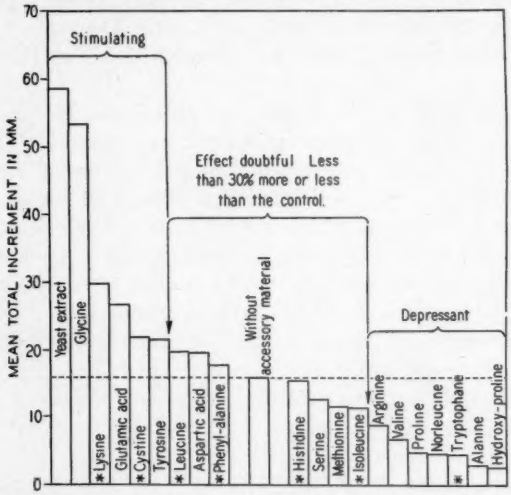

FIG. 1. Relative effectiveness of single amino-acids when tested at a concentration of 5 mg. per liter, in the absence of all other accessory materials. Those marked with an asterisk are considered by ROSE (9) to be essential for growth of rats.

studied proved to be depressant, and only 3—glycine, lysine, and glutamic acid—were definitely stimulating. Under these conditions glycine gave results almost equal to the control.

Optimal concentrations were then determined individually for each acid. For this purpose, each was tested at concentrations of 50, 15, 5, 1.5, 0.5, 0.15, and 0 mg. per liter of nutrient. Isoleucine, norleucine, methionine, serine, and arginine, which gave regularly increasing growth rates with decreasing concentration throughout this range, with the optimal growth better than the controls, were tested at additional concentrations of 0.05 and 0.015 mg. per

TABLE I

GROWTH INDICES OF ISOLATED TOMATO ROOT-TIPS AT VARIOUS CONCENTRATIONS OF SINGLE AMINO ACIDS. THE GROWTH RATE IN THE ABSENCE OF ALL ACCESSORY MATERIAL IS TAKEN AS 100

AMINO ACID	CONCENTRATION—MG. PER LITER								
	50	15	5	1.5	0.5	0.15	0.05	0.015	0
Glutamic acid	35	*133*	97	102	97	98	.....	.....	100
Aspartic acid	15	*124*	109	98	106	86	.....	.....	100
Glycine	38	84	*306*	146	97	104	.....	.....	100
Lysine	31	157	*216*	194	200	171	.....	.....	100
Phenyl-alanine	52	86	*118*	98	100	95	.....	.....	100
Proline	30	41	56	*134*	93	74	.....	.....	100
Cystine	70	70	100	*128*	104	100	.....	.....	100
Hydroxyproline	7	9	18	71	*127*	99	.....	.....	100
Leucine	20	50	110	114	*118*	103	.....	.....	100
Histidine	16	51	62	92	*109*	100	.....	.....	100
Valine	5	22	44	104	*108*	97	.....	.....	100
Methionine	34	42	48	67	100	*139*	100	.....	100
Serine	11	33	66	82	107	*111*	93	98	100
Norleucine	10	15	22	32	56	*128*	124	103	100
Arginine	11	26	74	110	110	*127*	100	.....	100
Tyrosine	19	42	76	99	84	*103*	.....	.....	100
Isoleucine	21	31	51	81	124	138	*174*	99	100
Tryptophane	13	20	36	78	85	86	.....	.....	100
Alanine	6	10	14	24	90	76	.....	.....	100

liter. This series included about 3000 individual cultures so that it was necessary to run them in 10 consecutive sets, each with a separate set of controls. In order to facilitate comparison of the sets within this series with one another, the results have been expressed as percentages of the controls. The results are shown in table I. All amino acids studied proved to be depressant at 50 mg. per liter (50 p.p.m.). Alanine, valine, hydroxyproline, and norleucine were quite definitely so. Tryptophane and alanine were depressant at all concentrations studied. Tyrosine was apparently not beneficial at any concentration tested. Valine, histidine, serine, phenyl-alanine, methionine, cystine, leucine, norleucine, arginine, aspartic acid, glutamic acid, proline, and hydroxyproline showed slight stimulation at some point in the concentration range investigated. Glycine, lysine, and isoleucine were markedly stimulating at favorable concentrations. The optimal concentrations varied from 15 p.p.m. in the case of glutamic and aspartic acids down to 0.05 p.p.m. in the case of isoleucine. A typical series of results is shown in figure 2 (glycine).

From these data an amino acid mixture having the provisional constitution shown in the first column of table II was chosen for study. MEISEN-

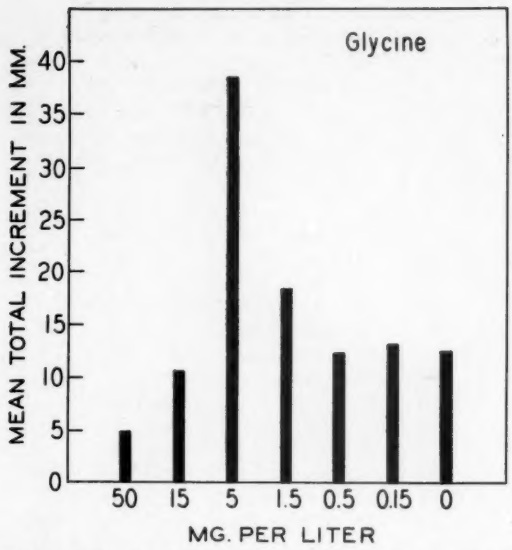

FIG. 2. Histogram showing total increments obtained in a week's time in nutrients containing various concentrations of glycine, without other accessory material.

HEIMER has studied in detail the nitrogenous constituents of hydrolyzed yeast (**2, 3**). Although his figures are much higher than would be the case for an alcohol extract, it is clear that such an extract could never contain *more* of any amino acid than he has indicated. His figures have, therefore, been recalculated to give comparable units and are presented for comparison.

The last column of the table is of especial interest. In most cases the experimentally determined optimum concentration for nutrition of isolated tomato root-tips did not differ widely from the amounts actually found in a hydrolyzed yeast extract. But the optimal amounts found for glycine, cystine, glutamic acid, and aspartic acid were so much higher than can possibly be present in yeast (MEISENHEIMER, see table II) that some explanation would seem necessary. It is to be noted that three of these—glycine, glutamic acid, and aspartic acid—are not among the nine amino acids recognized by ROSE (**8, 9**) as essential for animals. It seemed possible that in a complete mixture these three might prove to be partially or wholly interchangeable with some of the other amino acids. However, it is to be remembered that MUELLER (**5**) found diphtheria bacilli to require one of these, glutamic acid, in unexpectedly large amounts.

On the basis of the information contained in the data presented above, an amino acid mixture was prepared containing 15 mg. each of glutamic acid

TABLE II

CONCENTRATIONS OF SINGLE AMINO ACIDS OPTIMAL FOR GROWTH OF ISOLATED TOMATO ROOT-TIPS

AMINO ACID	OPTIMUM CONCENTRATION (a)	TOTAL CONCENTRATION IN OPTIMAL YEAST CONTENT (FROM MEISENHEIMER) (b)	RATIO a/b
	mg./liter		
Glycine	5.000	0.03	166.7
Cystine	1.500	0.04	37.5
Lysine	5.000	0.52	9.6
Glutamic acid	15.000	0.62	24.1
Isoleucine	0.050	0.23	0.2
Norleucine	0.150	.....	.....
Methionine	0.150	0.14*	1.1
Serine	0.150	1.14	0.1
Proline	1.500	0.16	0.9
Hydroxyproline	0.500	0.38	1.3
Phenyl-alanine	5.000	1.13	4.4
Leucine	0.500	0.70	0.7
Valine	0.500	0.84	0.6
Arginine	0.150	0.31	0.5
Aspartic acid	15.000	0.38	39.3
Histidine	0.500	0.24	2.1
Total	50.650	6.86	7.4

* MEISENHEIMER (2, 3) does not mention methionine by name but speaks of "sulfur containing amino acids other than cystine" as being present in this amount. It is assumed that the "amino acid" to which he refers was probably methionine.

and aspartic acid, 5 mg. of glycine, lysine, and phenyl-alanine, 1.5 mg. of cystine and proline, 0.5 mg. of hydroxyproline, leucine, histidine, and valine, 0.15 mg. of methionine, serine, norleucine, and arginine, and 0.05 mg. of isoleucine, totaling 50.65 mg. of amino acid in each liter of solution. Since this was about 7 times the total amount of amino acid contained in the optimal concentration of yeast (MEISENHEIMER, 2) and since concentrations, both relative and absolute, have repeatedly proved of great importance, the mixture was tested at 1, 0.3, 0.1, 0.03, and 0.01 times this concentration. The results are shown in figure 3. As was the case with single amino acids, a concentration of 50 mg. per liter was quite depressant, the optimum lying at about 15 mg. per liter, one-third the concentration originally chosen.

As has already been suggested, the importance of many of the amino acids used above, especially in the presence of the other amino acids, was subject to considerable doubt, since it is known that in animal nutrition cer-

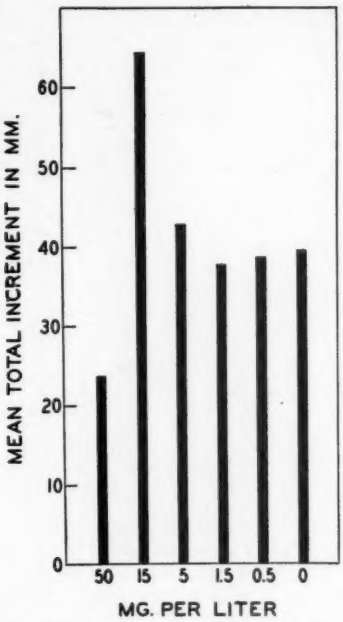

FIG. 3. Effects on growth of various concentrations of a mixture containing 16 amino acids (see text).

tain acids may substitute for one another (ROSE, *et al.*). It thus seemed probable that some of these amino acids, which when taken singly proved to be beneficial, might nevertheless be unnecessary for optimal growth. To test this, a series of cultures was made, in each of which one amino acid was employed at 10, 1, 0.1, and 0 times the concentration (one-third that shown in table II) found to be optimal in the last experiment, while all other amino acids were present at the optimal concentrations. This test involved 64 combinations, 1280 cultures. In each set of cultures a solution containing all amino acids except the one under examination was used as control. The results, given as percentages of these controls are presented in table III.

It will be seen from the table that of the 16 amino acids which have a stimulating effect at some concentration when taken alone, 7 fail to exert such an effect when in the presence of all the others. These 7 are glycine, aspartic acid, hydroxyproline, cystine, methionine, arginine, and norleucine. Reference to table II will show that of these, 3—glycine, cystine and aspartic acid—were among those which, when taken singly, gave optimum results at concentrations greater than those existing in the optimal concentration of yeast. It seems probable that the originally observed effects of these 3 (table

TABLE III

GROWTH INDICES OF ISOLATED TOMATO ROOT-TIPS GROWN IN A COMPLETE AMINO ACID MIXTURE (TABLE II) IN WHICH THE CONCENTRATIONS OF SINGLE AMINO ACIDS WERE VARIED. THE GROWTH RATE IN A MIXTURE, COMPLETE EXCEPT FOR THE AMINO ACID UNDER EXAMINATION, IS TAKEN AS 100

AMINO ACID	RELATIVE CONCENTRATION OF THE AMINO ACID WHOSE CONCENTRATION IS BEING VARIED. ONE-THIRD OF THE VALUE INDICATED IN TABLE II AS INDIVIDUALLY OPTIMAL IS TAKEN AS 1 (COLUMN 2) (SEE TEXT)			
	10	1.0	0.1	0
Histidine	*136*	115	108	100
Proline	85	*120*	102	100
Serine	88	*122*	104	100
Valine	82	*124*	114	100
Phenyl-alanine	48	*124*	*124*	100
Glutamic acid	35	*144*	*138*	100
Lysine	56	*162*	*159*	100
Leucine	80	96	*133*	100
Isoleucine	80	99	*118*	100
Arginine	*100*	*104*	*102*	*100*
Methionine	68	*98*	*96*	*100*
Cystine	60	*89*	*90*	*100*
Aspartic acid	14	72	*97*	*100*
Glycine	53	67	*89*	*100*
Hydroxyproline	63	80	*92*	*100*
Norleucine	61	82	77	*100*

II) may have been due to traces of other essential amino acids present as impurities in the samples studied, or else that they may function only as building blocks for other essential acids present in the complete mixture. A fourth amino acid, methionine, discarded by this process, is known to be a common contaminant in commercial preparations of leucine (MUELLER, 6) and was possibly supplied in the requisite amount in this material. Of the 4 acids giving aberrant results (table II), only glutamic acid remains as apparently essential *sui generis*. Using diphtheria bacilli, MUELLER likewise found the optimal concentration of glutamic acid to be abnormally high.

If the acids found to be essential for rats (ROSE, 9), for diphtheria bacilli (MUELLER, 5), and for tomato roots (this work) are tabulated, the very close correspondence between the three sets is evident. Only two amino acids—proline and serine—present in the list developed for tomato root-tips are not present in either ROSE's or MUELLER's lists. Four acids—cystine, methionine, glycine, and tryptophane—are to be found in one or both of these lists but not in the present one. Tryptophane alone, of those found essential by both ROSE and MUELLER, has under no circumstances appeared

beneficial for tomato root-tips. In view of its close chemical relationship to heteroauxin, which is known to have a depressant effect on root growth, tryptophane may have a place which is unique. Further study will be required before its true position can be considered established.

TABLE IV

AMINO ACIDS ESSENTIAL FOR OR BENEFICIAL TO ORGANISMS OF THREE WIDELY SEPARATED GROUPS

RATS (ROSE)	TOMATO ROOTS (WHITE)	DIPHTHERIA BACILLI (MUELLER)
Histidine	Histidine	Histidine
Phenyl-alanine	Phenyl-alanine	Phenyl-alanine
Lysine	Lysine	.....
Leucine	Leucine	.....
Isoleucine	Isoleucine	.....
Cystine	.....	Cystine
Tryptophane	.....	Tryptophane
Threonine*	(Not tested)	(Not tested)
.....	Valine	Valine
.....	Glutamic acid	Glutamic acid
.....	Proline	.....
.....	Serine	.....
.....	.....	Glycine
.....	.....	Methionine

* = α-amino-β-hydroxy-n-butyric acid (4).

A provisional amino acid mixture which appeared to be complete and possibly optimal then contained: 5 mg. of glutamic acid, 1.5 mg. each of phenyl-alanine, lysine, and histidine, 0.5 mg. of proline, 0.15 mg. of valine, 0.05 mg. of serine, 0.015 mg. of leucine, and 0.0015 mg. of isoleucine per liter of nutrient. The total amino acid content of this mixture was 10.2165 mg. per liter.

Since such an amino acid mixture was developed in the hope of replacing therewith the fraction of yeast soluble in 85 per cent. ethyl alcohol but insoluble in 100 per cent. alcohol, a series of cultures was carried out using this mixture together with a 100 per cent. alcohol extract of yeast in amount equivalent to the standard amount of whole yeast regularly used as control. The results are shown in figures 4 and 5. Such a mixture of amino acids, when added to the yeast material soluble in absolute alcohol, is adequate for the maintenance of continuous growth for at least three passages, at a level equal to or nearly equal to that obtained with whole yeast. The entire effect of the yeast material soluble in 85 per cent. alcohol but insoluble in absolute alcohol, insofar as it can be observed in this way, is then to be attributed to its amino acid content.

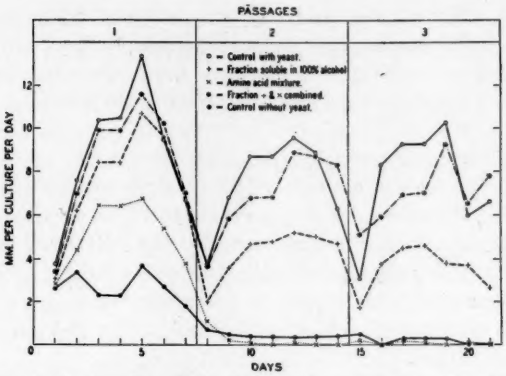

FIG. 4. Graph showing the effects during three passages[1] of a mixture of 16 amino acids (see text) when taken alone and in the presence of a 100 per cent. alcohol extract of yeast.

A B C D E

(*Photograph by* J. A. CARLILE)

FIG. 5. Reading from left to right: A, control with yeast extract. B, control without accessory material. C, amino acid mixture alone. D, yeast material soluble in 100 per cent. alcohol, insoluble in ether. E, same as D but with amino acid mixture (C) added. Photograph taken at the end of the third passage. ×1.

There remains to be identified only the yeast material soluble in absolute alcohol, representing 0.0005 per cent. of the total nutrient.

Summary and conclusions

In an earlier paper it was shown that yeast extract contains certain materials essential for growth of isolated tomato roots. These are all soluble

[1] "Passage 1" in this experiment is passage 196, counting from the time of isolation, the roots having been maintained through 195 passages under standard conditions before being used in this experiment.

in 85 per cent. ethyl alcohol, and insoluble in ether. Further extraction with 100 per cent. alcohol segregates two fractions, both of which are necessary for satisfactory growth. Data have been presented in this paper which show that the fraction which is soluble in 85 per cent. alcohol, but insoluble in 100 per cent. alcohol and ether, may be replaced by a mixture of 9 amino acids without appreciably reducing the growth rate. The amino acid content of this fraction appears to be entirely responsible for its growth-promoting effect, at least so far as the requirements of isolated tomato root-tips are concerned. Such a mixture contains the following: glutamic acid, lysine, histidine, phenyl-alanine, leucine, isoleucine, valine, serine, and proline. All other amino acids appear to be unessential under the conditions of the experiment reported. The fraction soluble in 100 per cent. alcohol remains to be identified.

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VITAMIN B_1 IN THE NUTRITION OF EXCISED TOMATO ROOTS

PHILIP R. WHITE

(WITH FIVE FIGURES)

Introduction

In previous studies of the growth-promoting materials obtainable from yeast and essential for the nutrition of excised tomato roots, these materials were shown to be soluble in 85 per cent. ethyl alcohol and insoluble in ether. Further extraction with absolute alcohol segregated two fractions both of which proved essential for optimal growth (**5**). The effectiveness of the insoluble residue left after absolute alcohol extraction of the 85 per cent. alcohol-soluble material was shown to be largely if not entirely attributable to the amino acids contained therein (**6**). The material soluble in absolute alcohol was not studied.

Besides the properties mentioned above, this material was shown to be stable to prolonged boiling in trichloroacetic acid, resistant to repeated drying, and resistant to moderately prolonged autoclaving such as was regularly employed in the preparation of nutrients (**4**). Further tests showed that its activity was completely destroyed by boiling for 2 hours in 1 N NaOH. Boiling for a similar period in 6 N HCl gave a product which supported apparently normal growth during the time necessary for a single passage (one week) but not for longer periods. The writer wishes to thank Dr. HUBERT S. LORING for carrying out these last treatments and for valuable advice during the conduct of the work.

The properties of this material as now known (solubility in 100 per cent. alcohol, destruction by alkali, and moderate stability in acid) are characteristic of vitamin B_1. It thus appeared possible that vitamin B_1 might be an important constituent of the fraction in question. Nevertheless, certain unpublished evidence obtained early in the conduct of this investigation seemed to oppose this view. Before any attempt was made to analyze the yeast material, a number of substances reported to act as growth stimulators was studied. These included inositol, vitamin B_1, heteroauxin, β-indolylpropionic acid, haemin, yeast nucleic acid, glutathione, and a number of different sugars. None of these substances gave any evidence of stimulating growth of isolated tomato roots when tested alone. The effort to approach the problem in this way was therefore abandoned.

Nevertheless, out of the work already published there has grown the conviction that failure to demonstrate the effectiveness of any material when studied alone cannot be taken as conclusive evidence of its ineffectiveness in a complete nutrient. It seemed possible, therefore, that vitamin B_1,

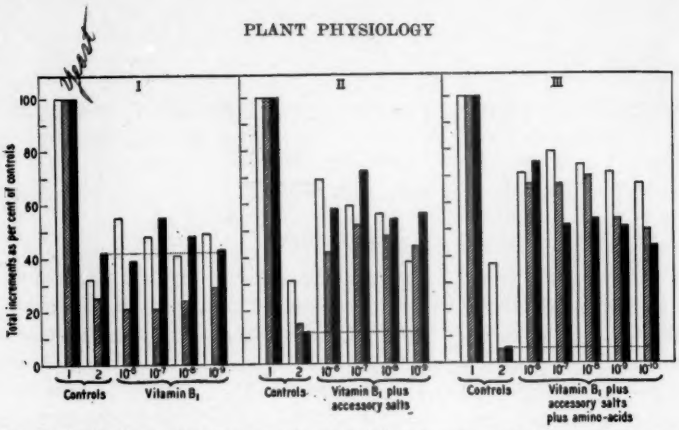

FIG. 1. Histograms showing growth of isolated tomato roots in nutrients containing standard salts, and sugar, with the addition in I of vitamin B_1 at concentrations of 10^{-6}[1] to 10^{-9}, of vitamin B_1 and accessory salts in II, and of these materials and amino acids in III. The open columns represent the first week's results (average of 20 cultures in each nutrient), the hachured columns the second week, and the blocked-in columns the third week. The transverse dotted line in each case is placed at the level of the negative control (without accessory organic material) in the third passage. All experimental increments must be above the level of this line to indicate growth stimulation. Control 1 is with yeast extract added to the basic nutrient, control 2 without accessory organic material of any sort.

although shown to be ineffective when taken alone, in the presence of the amino acid mixture developed in recently reported work (**6**) might prove to be of importance.

A study of the effects of vitamin B_1 in combination with these amino acids was, therefore, undertaken. While this work was under way the notes of BONNER (**1**) and of ROBBINS and BARTLEY (**2**) appeared, reporting evidence of the importance of vitamin B_1 in the nutrition of isolated roots. The present work is, therefore, a supplement to the work of these authors.

The basic nutrients, cultural methods, and materials used were the same as those previously described (**5, 6**). At the time these experiments were begun the roots were in the 196th passage.

Experimentation

Cultures were first carried out in the nutrient heretofore designated as basic, containing standard salts (including iron), accessory salts, and sugar (**5**), alone and with the following additions: amino acids only; vitamin B_1 only; amino acids and vitamin B_1 together; and yeast extract as control.

[1] This notation has been employed in the figures to conserve space. A concentration of 10^{-6} parts of vitamin B_1 in 1 part of nutrient is equivalent to 1 mg. per liter or 1 ppm. or 1 γ per cc.

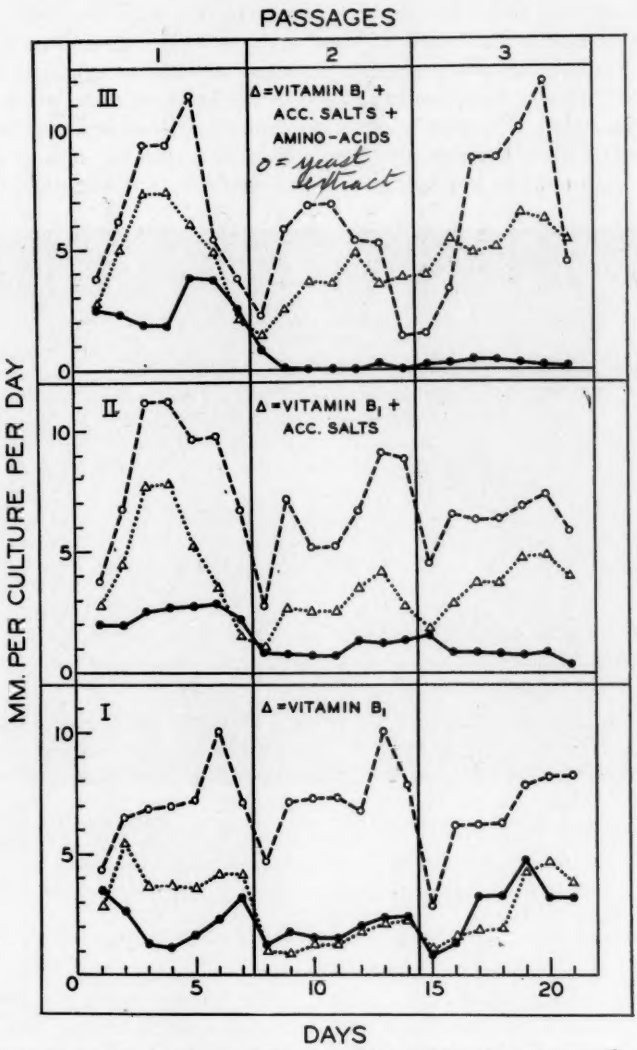

FIG. 2. Curves showing the growth rates of isolated tomato roots over three weeks' time in nutrients containing standard salts and sugar (solid circles) and with yeast extract added (open circles). The triangles represent growth in the same nutrient as that represented by the solid circles with the addition in I of vitamin B_1 at a concentration of 1.0 mg. per liter, of vitamin B_1 and accessory salts in II, and of vitamin B_1, accessory salts, and amino acids in III.

Vitamin B_1 was added at concentrations of 1.0, 0.1, 0.01, and 0.001 mg. per liter when tested alone, and at these and the additional concentration of 0.0001 mg. per liter when tested in combination with the amino acids.

When vitamin B_1 alone was added to the basic nutrient, a marked increase in growth rate occurred over the controls without accessory material. The results were, however, far from uniform or optimal (figs. 1–II, 2–II, and 4). In the first passage, growth at vitamin B_1 concentration of 1 mg.

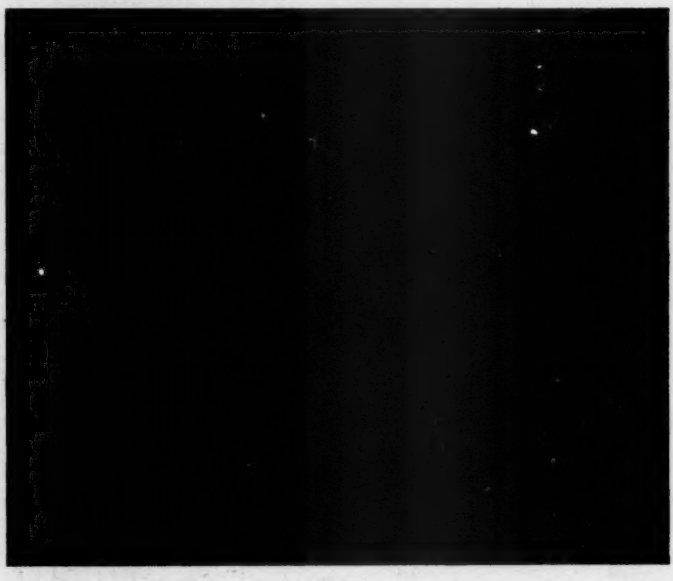

FIG. 3. Roots grown in a basic nutrient plus yeast (upper left), without any accessory material (upper right), and with addtiion of vitamin B_1 (lower row) at concentrations, reading from left to right, of 1.0, 0.1, 0.01, and 0.001 mg. per liter. Roots in the lower row are little if at all superior to the negative control. ×0.7.

per liter was equal to 69 per cent. of the control in yeast extract. The growth rates decreased regularly (59 per cent., 56 per cent., 38 per cent.) with decreasing concentration of vitamin B_1. In the second and third passages, a vitamin B_1 concentration of 0.1 mg. per liter gave the best results (52 per cent. and 72 per cent.). The roots were slender; most of the original growing points died so that growth occurred only in branches, and the branching was sparse. The condition of the cultures was obviously poor (fig. 4). A nutrient containing, in addition to the basic ingredients, only vitamin B_1, while capable of sustaining growth for a considerable period—

at least three passages—was clearly not satisfactory as a replacement for yeast extract.

This result agrees with that reported by ROBBINS and BARTLEY (**2**). The growth obtained was, however, much better than that observed two years ago, as mentioned above. In examining the records of the two sets of experiments for possible explanations of this discrepancy, two differences between the sets were noted. In the first place, the early tests were carried out with "crystalline vitamin B_1 Merck" of natural origin, while the later ones were conducted with "Betabion Merck," a synthetic product. In the second place, the later tests were made in a nutrient which included "accessory salts" (**5, 6**), while these were absent from the nutrient used (**4**) in the earlier tests. It seemed improbable that growth-promoting materials present in the synthetic vitamin B_1 might be absent from the natural product. The presence or absence of the accessory salts, therefore, seemed the more likely cause of the discrepancy.

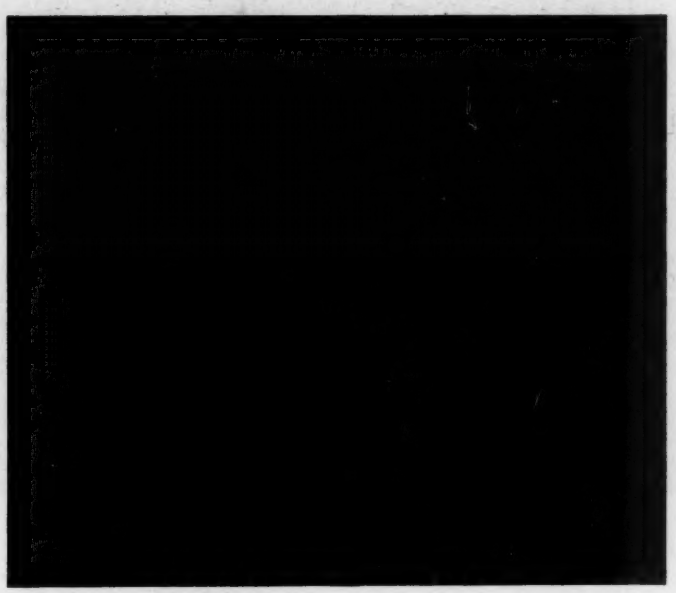

FIG. 4. Roots grown in nutrients similar to those used in cultures shown in figure 3, but with accessory salts added. Although the roots in the lower row are superior to the negative control (upper right), they are markedly inferior to those grown in the presence of yeast extract (upper left). It is clear that the tips in each experimental case had failed to grow and only a few branches were formed. $\times 0.7$.

To test this possibility, cultures were carried out in which vitamin B_1 was added at concentrations of 1.0, 0.1, 0.01, and 0.001 mg. per liter to a nutrient containing only the standard salts and sugar. In the first passage, while residual supplies of possible accessory salts carried over in the explant were still potentially available, the best growth, at a vitamin B_1 concentration of 1 mg. per liter, was equal only to 55 per cent. of the control (figs. 1–I, 2–I, and 3). In the second passage, growth at this concentration was only 21 per cent. of the control. In the third passage, growth at a concentration of 0.1 mg. per liter was equal to 55 per cent. of the control, but since the negative control, lacking all accessory material, likewise grew unusually well—42 per cent. of the control with yeast—this represents very little improvement over the completely deficient nutrient (figs. 2–I, and 3). From

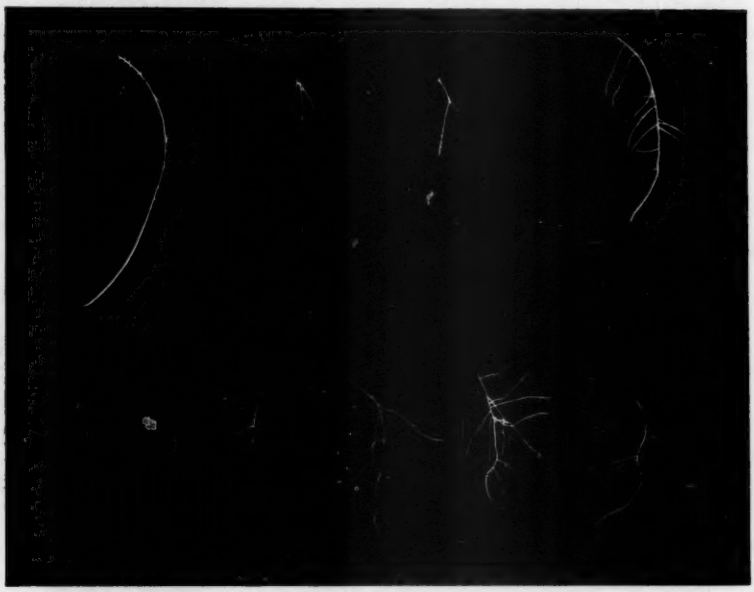

FIG. 5. Roots grown in the following nutrients: upper row, reading from left to right, control with standard salts, accessory salts, sugar, and yeast; the same but yeast omitted; the same but with an amino acid mixture substituted for the yeast; the same but with an absolute alcohol extract of yeast substituted for the usual yeast material; lower row, the same but with an amino acid mixture and vitamin B_1 at concentrations of 10^{-6} to 10^{-10} (from left to right) substituted for the yeast extract. The growing points of the experimental cultures have not died and the roots have branched quite profusely. While markedly superior to the roots grown without accessory organic material or with amino acids alone, or without accessory salts (fig. 3), they are still inferior to the controls provided with yeast. $\times 0.7$.

these results it is clear that the accessory salts were the *limiting* factor, the vitamin B_1 being unable to induce growth in their absence. The omission of accessory salts, therefore, was, as postulated above, responsible for failure to detect the effect of vitamin B_1 in earlier experiments. These results are in agreement with those of ROBBINS, WHITE, MCCLARY, and BARTLEY (**3**).

When vitamin B_1 was similarly added to a nutrient containing the 9-amino-acid mixture developed in earlier work (**6**) in addition to the basic nutrient, the results were somewhat better than in a similar nutrient lacking the amino acids, but still far from optimal (figs. 1–III, 2–III, and 5). In all concentrations except 1 mg. per liter there was a regular diminution in growth rate from passage to passage, while at this concentration the growth rates in the three passages studied (71 per cent., 67 per cent., 75 per cent. of the control) were remarkably similar. The roots, while still slender and of reduced vitality (fig. 5), were much more profusely branched than when amino acids were omitted. Their color was better and the original growing points did not die. This nutrient, containing standard salts, accessory salts, sugar, vitamin B_1, and amino acids, appeared to be capable of sustaining continuous growth of tomato roots at a level somewhat inferior to that obtained with yeast extract.

Discussion

The work presented here has demonstrated that vitamin B_1 is of paramount importance in the nutrition of isolated tomato roots. This material is known to be present in yeast and is soluble in 100 per cent. alcohol. It was, no doubt, at least partly responsible for the activity of the yeast fraction extracted by this solvent. While concentrations of vitamin B_1 as low as 0.0001 mg. per liter gave a marked degree of stimulation, concentrations of 1.0 or 0.1 mg. per liter appeared to be somewhat superior. Since the yeast fraction soluble in 100 per cent. alcohol gave optimal results at a concentration of about 5 mg. per liter (**5**)—only slightly greater than this amount—it would appear that this fraction might consist largely of vitamin B_1. Yet vitamin B_1 was *not* capable of completely replacing the fraction. Addition of amino acids (**6**) improved the result but still did not bring it up to the control. The roots cultivated in such a nutrient, while growing at a fairly uniform rate, were slender, crooked, and of reduced vitality. Some other material, soluble in 100 per cent. alcohol and effective at concentrations of less than 1 mg. per liter, while not *essential* for growth, must be responsible for the residual activity of this fraction.

The nutrient developed in this work, while inferior to yeast extract, does appear to be *complete,* in the sense of supporting *continuous* growth of tomato roots. And its constitution, except for possible impurities in the

"C.P. grade" chemicals used, is known and can be duplicated. One of the primary tasks in the study of growth requirements of isolated roots—the establishment of a completely known control nutrient containing all necessary growth factors—has then been provisionally completed. This nutrient, containing standard salts, accessory salts, sugar, vitamin B_1 at a concentration of 1.0 to 0.1 mg. per liter, and a 9-amino-acid mixture at a total concentration of 10 mg. per liter, may be used as a standard in future work.

While this nutrient appears to be "complete," its obvious deficiency when compared to a yeast extract medium raises certain important questions which must be left for future work to answer. Is the deficiency qualitative or quantitative? If it is only quantitative, a careful examination of the present ingredients of the mixture at higher and lower concentrations than at present used should serve to determine the optimal quantities. It is probable that in this process one or more ingredient out of the 18 salts and 9 amino acids at present included may prove to be unnecessary. If, however, as is equally likely, the deficiency is qualitative as well as quantitative, it will be more difficult to correct. The synthetic nutrient may be supplying materials which are capable, partly but not completely, of replacing some yeast material. The substitution of selenium for sulphur or of pyrrol for iron would be similar cases. If such is the case, the optimal concentrations of these "Ersatz-" materials would still give results inferior to those obtained with yeast extract. No amount of quantitative adjustment of the nutrient will correct the deficiency or even clearly indicate which ingredient is deficient, and more complex methods will have to be devised for solving the problem. To determine which is actually the case, a quantitative study of all the present ingredients of the nutrient is, therefore, indicated as the next step in approaching these questions.

Summary

Experiments have shown that vitamin B_1, a probable constituent of the yeast fraction soluble in absolute alcohol, is an important and perhaps indispensable factor in the nutrition of excised tomato roots. The growth-promoting effect of vitamin B_1 is detectable only in the presence of the "accessory salts" which are also indispensable. While growth at a low level can be maintained apparently indefinitely in a nutrient containing only vitamin B_1, standard salts, accessory salts, and sugar, it is notably improved by the addition of a mixture of 9 amino acids.

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DORMANCY AND GERMINATION OF *FRAXINUS* SEEDS

GEORGE P. STEINBAUER

(WITH SIX FIGURES)

Introduction

The genus *Fraxinus* contains several species of woody plants of economic importance. The majority of these are propagated from seed in nature and under cultivation. Very little specific information is available relative to the factors affecting seed germination in the different species.

Special treatments of the seeds are often required to produce uniform stands of seedlings in the year following harvest of the seed. The most common recommendations for germinating *Fraxinus* seeds suggest stratification of the seeds at 5°–10° C. previous to planting (**8, 11**). In many instances, untreated seeds may remain dormant in the soil for one or more years after planting. PUCHNER (**10**) reported one case in which seeds of European ash, *Fraxinus excelsior* L., remained in a dormant condition in the soil for six years before they germinated.

Information on the causes of delayed germination of *Fraxinus* seeds is fragmentary and meager. The most thoroughly studied case is that of the seeds of European ash (**4, 9**) in which the embryo is small, though morphologically complete at the maturity of the seed, and requires a period of time for enlargement before the enveloping tissues and coats can be broken. Mechanical resistance of the enveloping tissues has been suggested as a possible reason for further delay in germination. Studies on green ash seed have suggested a dormant condition of the embryo (**7**).

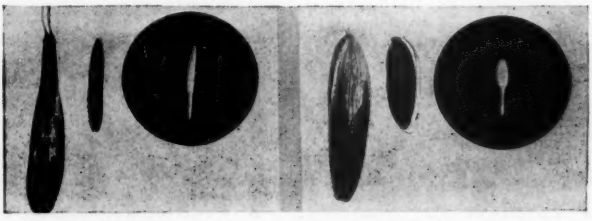
The present study has been confined to the following aspects of the problem: first, the relative importance of embryo and enveloping tissues in causing delayed germination; second, the factors affecting the growth of excised and encased embryos; third, the effectiveness of current stratification procedures in aiding germination of the seeds of different species; and fourth, the factors affecting the vitality of stored seeds.

Seeds of the following four species were used as material for this study: white ash, *Fraxinus americana* L.; black ash, *Fraxinus nigra* Marsh.; red ash, *Fraxinus pennsylvanica* Marsh.; and green ash, *Fraxinus pennsylvanica* var. *lanceolata* (Borkh.) Sarg. The above species are of commercial importance, grow in somewhat different habitats, and produce seed in the vicinity of Orono, Maine. The data presented here should be considered as applying primarily to the species studied, although there is some evidence that these results may apply also to other species.

Observations

Structure of the seed and fruit of *Fraxinus*

Since the course of the germination process depends largely upon the structure of the seed, a brief description of it is essential. In *Fraxinus* the unit commonly spoken of as the "seed" is in reality a winged fruit or samara. The true seed is exposed only after the ovary wall or pericarp has been removed. The fruits, seeds, and embryos of red and black ash are shown in figure 1.



A B

Fig. 1. The fruit, seed, and embryo of *Fraxinus*. A. Red ash. B. Black ash. From left to right: fruit, seed, embryo. Approximately normal size.

The extent and location of the more important parts of the samara are shown diagrammatically in figure 2, using the fruit of black ash as an example. Usually one seed is produced within each fruit although two and even three seeds are not uncommon.

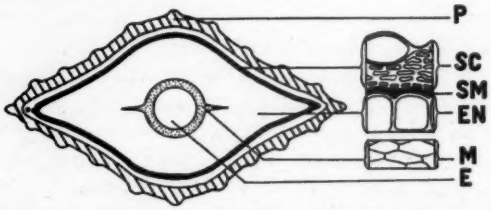


Fig. 2. Cross section of the fruit of black ash (diagrammatic). At the right a few cells are shown from the layers of the seed external to the embryo. Cell contents have been omitted.

P—pericarp
SC—seed coats
SM—suberized membrane
EN—endosperm
M—mucilaginous layer
E—embryo

The seed coats, SC, are made up of five to eight layers of cells. The innermost cells of the seed coats are separated from the endosperm cells, EN, by a membrane, SM. The exact chemical composition of the latter has not been determined although staining properties, solubility, and other microchemi-

cal tests indicate the presence of some cutin and suberin. This membrane is one of the most resistant layers external to the embryo, and very likely plays an important part in preventing rapid expansion of the embryo.

The reserve food materials of the endosperm consist largely of fats and reserve proteins. Starch and soluble carbohydrates appear in the endosperm and embryo in the later stages of the germination process.

The embryo, E, is surrounded by a layer of cells, M, containing mucilaginous material, called the "schleimschicht" by LAKON (9) relative to the seeds of European ash. These cells have thinner walls and less reserve food material than those of the endosperm. Owing to the semi-gelatinous consistency of this layer, the embryos of *Fraxinus* seeds may be easily excised without injury.

The degree of embryo development at maturity of the seed varies considerably among the different species. The embryos are fully differentiated into hypocotyl, cotyledons, and epicotyl, but the relative size of embryo and seed varies widely. In figure 1A there is illustrated the type of seed in which the embryo extends the full length of the seed. The seeds of red, white, and green ash are of this type. Such seeds are quite easily germinated if they are first stratified for a period of one or two months at a temperature near 5° C. In figure 1B there is illustrated the type of seed in which the embryo extends from one-half to two-thirds the length of the seed. Seeds of European ash are said to be similar to those of black ash in this respect (9). Such seeds do not germinate readily. They do not respond favorably to ordinary stratification treatments and in nature often remain in a dormant condition in the soil for one or more years before they germinate (5, 9).

DORMANCY AND GROWTH OF EXCISED EMBRYOS OF BLACK ASH

Since the embryos of black ash are much smaller in proportion to the rest of the seed than those of the other species studied, it seemed desirable to make observations on them first.

In order to determine whether or not embryos of recently harvested seeds are dormant, a number of embryos were excised from soaked seeds. They were placed on moist blotting paper in Petri dishes and kept at room temperature. At the end of two weeks there was no evidence of growth in such excised embryos.

Various treatments were then used in an attempt to overcome this initial dormancy. These treatments included different rates of aeration, use of light, various substrata, and a series of different temperatures. The use of higher than room temperatures was effective in inducing growth. Thus at the end of four days some of the embryos in the 30°-C. chamber were beginning to show geotropic curvature of the hypocotyl. This was followed by elongation of the cotyledons and the formation of root hairs. When these

seedlings were planted in soil in an illuminated moist chamber, they soon formed chlorophyll. Owing to the limited supply of reserve food contained in the embryo, they remained in a dwarfed condition for some time but eventually developed into normal plants.

The necessity for higher-than-room temperatures in breaking this initial dormancy is evident from table I, which shows the results obtained when lots of sixty embryos each were kept at temperatures of 5°, 20°, 25°, and 30° C. It is evident that dormancy is not equally deep seated in all embryos held at a given temperature. The results explain, in part, why stratification at low temperatures, which is so successful with some species of *Fraxinus*, is ineffective for seeds of black ash.

TABLE I

EFFECTIVENESS OF VARIOUS TEMPERATURES IN BREAKING THE DORMANCY OF EXCISED EMBRYOS OF BLACK ASH, *FRAXINUS NIGRA*

DURATION OF TEST	NUMBER OF EMBRYOS SHOWING GEOTROPIC CURVATURE (60 EMBRYOS BASIS OF RESULTS AT EACH TEMPERATURE)			
	5° C.	20° C.	25° C.	30° C.
days				
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	0	0	0	6
5	0	0	0	6
6	0	0	0	8
7	0	0	0	10
8	0	0	0	16
9	0	0	0	22
10	0	0	0	24
15	0	0	8	44
20	0	0	12	49
25	0	0	18	60
30	0	4	18	60

GROWTH OF ENCASED EMBRYOS OF BLACK ASH SEEDS

Although it is possible to produce plants from excised embryos of recently harvested seed, in nature the embryos undergo considerable enlargement before visible evidence of germination appears. LAKON (9), who reported a similar type of embryo development in the seeds of European ash, suggested the use of the term "Vorkeimung" to distinguish the growth changes of such embryos from the chemical and physical changes involved in "after-ripening" of seeds. The relative sizes of embryos at the beginning and end of this growth period are shown in figure 3. It is obvious that the small em-

bryos of recently harvested seeds are not comparable to the rudimentary embryos described by CROCKER (1) in *Corydalis, Ranunculus,* and other seeds. Since the time required for embryo enlargement may be weeks, or even months, it is important to know the optimum conditions for growth of the embryo.

FIG. 3. Enlargement of the black ash embryo previous to germination. Left, embryo at time of shedding of the seed. Right, embryo just previous to protrusion of the radicle in germination. About 2 × normal size.

The length of time required for the embryos to attain their maximum size depends to a large degree upon the temperature of the germination

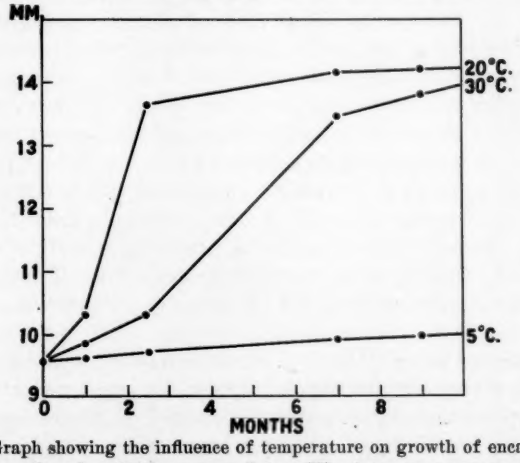


FIG. 4. Graph showing the influence of temperature on growth of encased embryos of black ash. Lengths of embryos expressed in millimeters are averages of one hundred measurements at each temperature.

chamber. Seeds were placed between layers of moist linen toweling and stored at temperatures of 5°, 20°, 25°, and 30° C. Embryos were excised from these different lots of seed at the end of four, ten, twenty-eight, and thirty-six weeks and measured for increases in length. The results obtained are shown graphically in figure 4. Measurements made on embryos held at 25° C. were intermediate between those of the 20° and the 30° C. groups and have been omitted from the graph.

The growth curve during this period of embryo development exhibits three rather distinct phases. The slow growth at first may be accounted for by the initial embryo dormancy mentioned above. This is followed by a period of rapid elongation of both cotyledons and hypocotyl. When the embryos have attained a length of 14–14.5 mm., the rate of growth rapidly decreases. Maximum size is attained at between two and three months at 20° C., and considerably later at 25° and 30° C. Further storage of seeds containing enlarged embryos at temperatures of 20° C. or higher does not ordinarily result in germination. One lot of seed stored at 20° C. had fully enlarged embryos at the end of two months and yet failed to produce a single seedling during the following two years of storage at 20° C.

That germination of seeds with fully enlarged embryos is not due to embryo dormancy is shown by the behavior of excised embryos. The latter will often show marked geotropic curvature within twelve hours after excision, which is soon followed by elongation of cotyledons and the formation of root hairs. By making a longitudinal incision in the seed, being careful not to injure the embryo, one can often induce germination within a few days. This suggests that the delay in germination is due to restrictions placed upon the embryo by the enveloping layers: endosperm, suberized layer, and seed coats. The pericarp probably is not an important factor since it disintegrates readily at temperatures of 20° C. or higher.

The retarding influence of the enveloping tissues on further growth of the embryo seems to be primarily in the nature of mechanical resistance. In this connection it is interesting to note that further growth of the cotyledons is not retarded as much at the higher temperatures as is the growth of the hypocotyl. In this respect the embryos are somewhat similar to the embryos of *Crataegus* (3) and *Ambrosia* (2). Abnormal germination is not uncommon in the seeds of black ash and in other species of ash. The appearances of *Fraxinus* seeds exhibiting normal and abnormal germination are shown in figure 5.

It is interesting to note that 5° C. is not conducive to embryo enlargement although it is a very effective temperature in forcing germination after the embryos have become fully enlarged. Likewise it is interesting to note that whereas 30° C. is a highly effective temperature for the growth of excised embryos it is less effective than 20° C. when the embryos are still within the seed.

FIG. 5. Normal and abnormal germination of *Fraxinus* seeds. Left, abnormal germination of red ash seed. Right, normal germination of white ash seeds. Note that in abnormal germination the cotyledons protrude before the hypocotyl whereas in normal germination the reverse takes place.

GERMINATION OF SEEDS CONTAINING FULLY ENLARGED EMBRYOS

Since temperatures favorable to enlargement of the embryo fail to promote normal germination, seeds with fully enlarged embryos were stored in a moist condition at temperatures of 5°, 20°, 30° C. and observed as to further changes in the embryos. Microchemical studies on seeds stored at these different temperatures indicated that 5° C. was the most favorable temperature for the various processes of digestion. Seeds stored at this temperature were characterized by a more rapid accumulation of starch, soluble carbohydrates, and proteins in the embryo than was the case with embryos of seeds stored at higher temperatures.

Excised embryos taken from these different lots of seeds also showed considerable variation in their capacity to resume growth. These differences are shown in figure 6. In this experiment embryos excised from seeds held at 5° C. exhibited geotropic curvature within ten hours after excision. Embryos from seeds stored at 20° C. required 24 hours to reach the same stage of development, and those from the 30° C. lot of seed were still in their original condition at the end of four days.

Although protrusion of the radicle occurs only rarely at 5° C., the digestion of reserves of the endosperm and the accumulation of these materials in the embryo make possible rapid growth and germination when the seeds are transferred to higher temperatures.

The following scheme summarizes the principal events leading up to the protrusion of the radicle through the seed and fruit coats. Optimum temperatures for the different stages of the germination process are indicated.

Seeds with small, morphologically complete embryos.
Dormancy of excised embryos broken by temperatures of 25°–30° C.

↓

I

EMBRYO ENLARGEMENT

Most effective temperature: 20° C. for 2–3 months.
Moderate rate of growth of excised embryos at this temperature.
Digestion slow.
Very little accumulation of food in embryo.

Enlargement of embryo ↓

Restrictions placed on further growth by enveloping tissues and membranes.
Mechanical resistance and possibly other factors involved.
Excised embryos not dormant. Normal germination rare at this temperature. Abnormal germination occasionally occurs.

↓

II

CHANGES IN SEED PRODUCED BY LOW TEMPERATURE STRATIFICATION

Most effective temperatures: 5°–10° C. for 2–3 months.
Growth of excised embryos very slow at this temperature.
Digestion continues. Depletion of fat and other reserve foods of endosperm. Accumulation of starch, soluble carbohydrates, and proteins in embryo.
Excised embryos not dormant. Germination may occur in seeds with more vigorous embryos.

↓

III

GERMINATION

Most effective temperatures: Daily alternation of 20°–30° C.
Growth of excised embryos rapid at this temperature.
Supply of food accumulated in embryos in stage II combined with high temperatures makes possible sufficient pressure (osmotic, imbibitional, etc.) to overcome mechanical resistance of layers external to embryos.

Seedlings may be produced from excised embryos at either stage, most easily at late I or in II.

FIG. 6. Effect of storage temperatures on behavior of excised embryos of black ash seeds. Left, embryos from 5° C. chamber. Center, embryos from 20° C. chamber. Right, embryos from 30° C. chamber.

GERMINATION OF SEEDS OF RED, GREEN, AND WHITE ASH

Studies on seeds of red, green, and white ash indicated that embryos excised from freshly harvested seed are dormant. In the case of red and green ash the period of dormancy can usually be reduced to a few days if the temperature of the germination chamber is maintained near 30° C. Embryos excised from white ash seeds remained dormant over a longer period of time and required a lower temperature for resumption of growth than did the embryos of the other species that were studied.

The most favorable conditions for germination of the seeds of red, green, and white ash are quite different from those required by seeds of black ash. Placing the seeds in germinators at temperatures at or near room temperature was ineffective in producing uniform germination. Abnormal germination as previously described occasionally occurs. The seeds of these species require approximately the same temperatures for germination as do the seeds of black ash at the time when the embryos have become fully enlarged. Thus the most uniform results are obtained when the seeds are stored in a moist condition for two or three months at 5°–10° C. and then transferred to a chamber with a higher temperature such as 20° C. or 30° C.

SUGGESTED PLANTING PRACTICES

The most uniform stands of seedlings can be obtained in the nursery or plantation by taking into account the temperature requirements for germination of the different species. In the case of red, white, and green ash very good results were obtained by fall planting immediately after the seeds had been harvested. This procedure allows the seeds to be subjected to moisture and low temperature during fall and early spring.

Occasionally the same results may be obtained if seeds that have been stored over winter in a dry condition are planted out of doors very early in the spring, so as to take advantage of the low temperatures then prevalent. If planting of dry seeds is delayed too long, the warm weather may prevent germination during the same season. If fall or early spring planting is not practiced, stratification of the seeds for two or three months at 5°–10° C. previous to planting is the best procedure.

Successful propagation of black ash seedlings from seed must take into account the conditions required for enlargement of the embryo. Fall plantings of black ash seed in the Maine State Forestry Nursery at Orono, Maine, have so far been unsuccessful in producing seedlings the following season. Germination occurred in the second spring after the planting of the seeds.

The planting of dry seeds in the spring is almost certain to result in failure to obtain a crop of seedlings until the following season. Stratification procedures commonly recommended for red, green, and white ash seeds should be preceded by a period of two or more months' storage of the seeds at a temperature near 20° C. This allows the embryos to become fully enlarged, after which the seeds may be treated in the same manner as those of red and green ash.

Effect of storage conditions on vitality of ash seeds

Since it is often desirable to defer planting of the seeds until the spring following the harvesting of the seeds, or occasionally to store them for more than one year, it is important to know the optimum storage conditions. The seeds of the majority of plants retain their vitality best when kept dry and at a low temperature. A few seeds, such as those of the maple (**6**), require a considerable amount of moisture along with a favorable temperature in order to remain viable.

In order to determine the most favorable storage conditions, aliquot samples of each of the different kinds of ash seed were stored in sealed containers. In one series the seeds were placed in sealed containers over dehydrating agents so selected as to yield various known relative humidities. In the second series the seeds were kept at their original moisture contents but at temperatures ranging from 5°–30° C. The results of these different treatments in terms of percentage of germination in the nursery beds are given in table II. Results for green and white ash seeds were similar to those for red and black ash seeds.

It is evident from the table that the seeds of these species are much more sensitive to changes in moisture content than to changes in temperature. Desiccation of the seeds to within 1.5 per cent. of their oven-dry weight did not appear injurious. The critical relative humidity of the storage chamber appears to be between 50 and 75 per cent. If the moisture content of the seeds can be reduced to about 7.5 per cent. of their dry weight and the seeds stored in closed containers, or if the storage room can be kept at a relative humidity of 50 per cent. or less, the seeds may be expected to retain their vitality for at least one or two seasons after harvest. Studies on excised embryos indicated that the reduction in the percentage of germination resulting from storage at high relative humidities was due to a deterioration of the embryo rather than to changes in the enveloping tissues and membranes.

TABLE II

VITALITY OF *FRAXINUS* SEEDS IN RELATION TO STORAGE CONDITIONS

STORAGE CONDITIONS*		BLACK ASH *FRAXINUS NIGRA*		RED ASH *FRAXINUS PENNSYLVANICA*	
		MOISTURE CONTENT	GERMINATION†	MOISTURE CONTENT	GERMINATION†
		%	%	%	%
Over concentrated sulphuric acid	25° C.	1.72	38	1.68	53
25 per cent. relative humidity	25° C.	5.55	46	5.36	48
50 " " " "	25° C.	7.33	39	6.49	50
75 " " " "	25° C.	11.39	0	9.85	1
5° C.		7.50	40	7.30	54
20° C.		"	50	"	49
25° C.		"	43	"	56
30° C.		"	38	"	51

* Storage of seeds for one year in sealed containers previous to planting.

† Figures based on quadruplicate samples of 100 seeds each.

Summary

1. A study of the optimum conditions for germination of the seeds of red, green, white, and black ash indicates that the seeds of black ash do not germinate at temperatures favorable to germination of the other species.

2. The differences in temperature requirements of the various species of ash depend in part upon the size of embryo at maturity of the seed.

3. Embryos of black ash seeds undergo considerable enlargement before germination occurs. Temperatures near 20° C. are most effective in promoting growth during this period.

4. Germination of the seeds of black ash does not occur at temperatures favorable to enlargement of the embryo. Further growth of the embryo is retarded by mechanical resistance of the enveloping tissues and coats.

5. Stratification of black ash seeds at 5° C. enables the fully enlarged embryos to absorb and accumulate reserve materials from the endosperm. After a period of two or three months stratification, the embryos are able to overcome the resistance of the enveloping membranes when placed at higher temperatures.

6. Embryos of red, green, and white ash are fully enlarged at maturity of the seed. Stratification of the seeds at 5° C. brings about changes favorable to germination.

7. Dwarf seedlings may be obtained from embryos excised from freshly harvested seeds of all of the species studied.

8. Storage of seeds in sealed containers at different temperatures and relative humidities showed that vitality of the seeds is retained the longest

when the seeds are kept at a moisture content of less than 7.5 per cent. of the dry weight. Seeds with a moisture content of less than 7.5 per cent. retained their vitality equally well over a wide range of temperatures.

9. Suggestions are given for effective planting procedures for the different species studied.

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INFLUENCE OF HOST NUTRITION ON SYSTEMIC DEVELOPMENT OF TOBACCO MOSAIC

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In two previous papers it was shown (**3, 4**) that the mineral nutrition of tobacco plants had a marked effect on their susceptibility to primary infection with a yellow strain of tobacco-mosaic virus. In the present paper the influence of host nutrition on systemic infection of this same virus in infected tobacco plants will be discussed.

Several workers have studied the movement of virus in plants, but little attention has been given to the influence which host nutrition may have on systemic spread of virus. BÖNING (**1**), in 1928, published the results of an experiment dealing with the effect of nitrogen nutrition on mosaic-virus spread in tobacco. The tip of the largest leaf on each plant was inoculated. With actively growing plants an average of 3 days was required for the virus to pass 13 cm. from the tip of the leaf to the stem. With slow-growing, nitrogen-deficient plants an average of at least 6 days was necessary for the virus to pass from a leaf 12 cm. long. BÖNING concluded that the rapidity of virus-spread from an inoculated leaf was greatly decreased by nitrogen deficiency. VOLK (**5**) studied the effect of potassium, phosphorus, and nitrogen on the spread of streak in tomato plants grown in nutrient sand cultures, and inoculated just prior to fruiting. He concluded that the spread of streak disease was most rapid and the effect of the disease most severe in plants fertilized with an ample supply of potassium and phosphorus or in plants deficient in nitrogen.

Experimentation

MATERIALS AND METHODS

Turkish tobacco (*Nicotiana tabacum* L.) was used as the experimental host. Seeds were germinated in quartz sand and kept moist with a dilute nutrient solution. When the seedlings had reached a height of about 2 cm. and had developed 3 or 4 small leaves, plants were selected for uniformity of size and potted in 4-inch porous clay pots filled with washed, quartz sand. The pots were then placed in saucers on a greenhouse bench and were spaced so as to avoid undue crowding of foliage. During the course of the experiments the greenhouse temperature was held between 70° and 80° F. during the day and between 70° and 75° F. at night. The greenhouse was fumigated with nicotine at weekly intervals to prevent accidental inoculation by insect vectors.

Nutrient treatments were usually started 3 days after the seedlings had been potted. Each seedling received 100 cc. of nutrient solution 3 times each

TABLE I

COMPOSITION OF NUTRIENT SOLUTIONS

NUTRIENT SOLUTIONS	VOLUME OF 0.5 MOLAR STOCK SOLUTIONS REQUIRED TO PRODUCE 1 LITER OF NUTRIENT SOLUTION							
	KH_2PO_4	$Ca(NO_3)_2$	$MgSO_4$	$(NH_4)_2SO_4$	$NH_4H_2PO_4$	KNO_3	K_2SO_4	$CaCl_2$
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
Nitrogen								
Minus	12.9	.....	4.0	.....	.....	.....	.....	7.8
Medium	12.9	7.8	4.0	3.6	.....	.....	.....	.....
High	.....	96.5	4.0	33.5	12.9	12.9	.....	.....
Phosphorus								
Minus	.....	11.0	4.0	3.3	.....	.....	48.4	.....
Medium	12.9	11.0	4.0	3.3	.....	.....	42.0	.....
High	96.8	11.0	4.0	3.3	.....	.....	.....	.....
Potassium								
Minus	.....	7.8	4.0	.....	12.9	.....	.....	.....
Medium	12.9	11.0	4.0	3.6	.....	.....	.....	.....
High	12.9	7.8	4.0	6.5	.....	.....	121.4	.....

week. The composition of these solutions is given in table I. In each case the "medium" solution served as a check on the "minus" and "high" solutions and was of such a composition as to produce healthy, vigorous growth. The "high" solution contained the element in question at such a level that growth was retarded but not entirely inhibited. The "minus" and "high" solutions were used in order that a study could be made of the action exerted not only by a deficiency but also by an excess of the element in question. In addition to the salts listed in table I, boron as H_3BO_3, and manganese as $MnSO_4.2H_2O$ were added in concentrations equivalent to 0.5 p.p.m. of each element to all culture solutions.

In each experiment the nutrient treatment was as follows: The seedings were divided into 7 groups. One group received the "medium" solution throughout the time of nutrient application. During the first week of nutrient treatment, 1 of the 6 remaining groups received the "high" solution and 5 received the "minus" solution. At the end of each week one group of seedlings was transferred from the "minus" to the "high" solution. Thus, at the time of inoculation, 4 weeks after the initial nutrient treatment, one group had received the "high" solution for 1 week, a second group for 2 weeks, a third group for 3 weeks, and a fourth group for 4 weeks. This arrangement left 2 groups on the "minus" solution, but at the time of inoculation 1 of these 2 groups was transferred to the "high" solution. All groups received their respective nutrient solutions for 1 week following inoculation and then were watered whenever necessary with tap water until the experiment was terminated. At time of inoculation, the green weight and height of representative plants in each group were determined in order to obtain criteria of comparative growth.

The virus used was a yellow strain of tobacco-mosaic virus, formerly designated by JOHNSON (**2**) as tobacco virus 6. Representative plants in each treatment were inoculated by rubbing the tip portion of a leaf situated about half-way up the stem with undiluted juice from a diseased tobacco plant. All plants inoculated showed many yellow primary lesions on the inoculated leaves, but no attempt was made to count them. Following inoculation, the time required for the virus to reach and produce symptoms on leaves at the top of the plant was noted. When systemic infection appeared shortly after inoculation, the first symptom was a clearing of veins. When systemic infection appeared only after many days, the first symptoms were yellow lesions with indistinct, irregular margins. This stage was followed by clearing of veins.

INFLUENCE OF NITROGEN NUTRITION ON SYSTEMIC INFECTION

The nitrogen series was started in September and extended into November, 1936. In this series the nutrient solutions, the compositions of which are given in table I, all contained 20 mg. of phosphorus and 25 mg. of potassium per 100 cc. of solution. The medium-nitrogen solution contained 16 mg. of nitrogen, and the high-nitrogen solution 200 mg. per 100 cc. of solution. Each of the 7 groups contained 48 plants. After 4 weeks of nutrient treatment, 30 representative plants were selected in each group and inoculated as previously described. Table II gives the experimental data obtained in this test on the influence of nitrogen nutrition on growth and systemic infection of the virus. The green weights, based on an average

TABLE II

EFFECT OF NITROGEN ON SYSTEMIC SPREAD OF A YELLOW STRAIN OF TOBACCO-MOSAIC VIRUS

NITROGEN ADDED EACH WEEK (MG./POT)					GROWTH RECORDS AT TIME OF INOCULATION*		PERCENTAGE OF PLANTS SHOWING SYSTEMIC INFECTION	NUMBER OF DAYS ELAPSED BEFORE APPEARANCE OF SYSTEMIC SYMPTOMS
1ST WEEK	2ND WEEK	3RD WEEK	4TH WEEK	5TH WEEK	GREEN WEIGHT	HEIGHT		
mg.	*mg.*	*mg.*	*mg.*	*mg.*	*gm.*	*cm.*	*%*	*days*
0	0	0	0	0	1.4†	1†	100	6.43‡ ± 0.26§
0	0	0	0	600	1.4	1	100	5.03 ± 0.03
0	0	0	600	600	4.9	2	100	5.00 ± 0.00
0	0	600	600	600	7.0	3	100	5.10 ± 0.06
0	600	600	600	600	14.6	8	100	5.77 ± 0.09
600	600	600	600	600	15.9	9	100	6.43 ± 0.21
48	48	48	48	48	32.7	28	100	21.27 ± 0.93

* Plants inoculated at end of 4th week.
† Average of 10 plants in each group.
‡ Average of 30 plants in each group.
§ Standard error of the mean.

of 10 plants in each group and determined at time of inoculation, show that the plants which received the minus-nitrogen solution made very little growth. Plants which received 600 mg. of nitrogen each week for 3 or 4 weeks made much better growth, although they were only about one-half the size of those which received 48 mg. of nitrogen each week. All inoculated plants, with the exception of those which received 48 mg. of nitrogen each week, showed symptoms of systemic infection within 10 days. There were only slight differences among the groups which received no nitrogen or an excess of nitrogen in the time required for the development of systemic symptoms. When no nitrogen was added, an average of more than 6 days elapsed before the development of such symptoms. When the high-nitrogen solution was first added at time of inoculation, an average of 5 days elapsed before the appearance of symptoms of systemic infection. Even when 600 mg. of nitrogen were added each week for 4 weeks prior to inoculation, about 6 days elapsed before the appearance of symptoms. However, when 48 mg. of nitrogen were added each week, an average of more than 21 days elapsed before symptoms of systemic infection developed. From these data it is apparent that the absence of nitrogen or the presence of relatively large quantities of nitrogen in the nutrient solution increased the rapidity of development of symptoms of systemic infection.

Influence of phosphorus nutrition on systemic infection

The phosphorus series was carried out during March and April, 1936. The compositions of the nutrient solutions used are given in table I. Each of these solutions contained 20 mg. of nitrogen and 190 mg. of potassium per 100 cc. of solution. The medium-phosphorus solution contained 20 mg. of phosphorus, and the high-phosphorus solution 150 mg. per 100 cc. of solution. Each of the 7 groups contained 48 plants. Thirty-six plants in each group were inoculated as previously described. The experimental data obtained in this study of the effect of phosphorus nutrition on growth and systemic infection are shown in table III. From the green-weight data, based on an average of 12 plants in each group, it is apparent that growth increased with the increasing period of time during which the high-phosphorus solution was added, but in no case did plants which received 450 mg. of phosphorus each week make as good growth as those which received 60 mg. each week. All inoculated plants showed symptoms of primary infection at the site of inoculation, but several of them failed to show symptoms of systemic infection. The data in the last column in table III give the average number of days which elapsed before the development of symptoms on the 28 plants which first showed systemic infection in each group. The phosphorus-deficient plants showed systemic infection in about 7 days. Plants that had received 450 mg. of phosphorus during the week prior to

TABLE III

EFFECT OF PHOSPHORUS ON SYSTEMIC SPREAD OF A YELLOW STRAIN OF TOBACCO-MOSAIC VIRUS

PHOSPHORUS ADDED EACH WEEK (MG./POT)					GROWTH RECORDS AT TIME OF INOCULATION*		PERCENTAGE OF PLANTS SHOWING SYSTEMIC INFECTION	NUMBER OF DAYS ELAPSED BEFORE APPEARANCE OF SYSTEMIC SYMPTOMS
1ST WEEK	2ND WEEK	3RD WEEK	4TH WEEK	5TH WEEK	GREEN WEIGHT	HEIGHT		
mg.	*mg.*	*mg.*	*mg.*	*mg.*	*gm.*	*cm.*	%	*days*
0	0	0	0	0	11.6†	7†	100	7.18‡ ± 0.17§
0	0	0	0	450	11.6	7	100	6.46 ± 0.11
0	0	0	450	450	16.0	7	100	7.75 ± 0.17
0	0	450	450	450	20.1	10	97	12.29 ± 0.78
0	450	450	450	450	28.3	15	97	16.79 ± 0.62
450	450	450	450	450	33.2	19	78	17.96 ± 0.82
60	60	60	60	60	38.6	24	81	19.36 ± 0.88

* Plants inoculated at end of 4th week.
† Average of 12 plants in each group.
‡ Average time for development of symptoms on the 28 plants which first showed systemic infection in each group.
§ Standard error of the mean.

inoculation showed systemic infection in about 8 days. Those that had received 450 mg. of phosphorus each week for 2 weeks prior to inoculation showed systemic infection in about 12 days. Those that had received this application of phosphorus for 3 weeks prior to inoculation showed systemic infection in about 17 days. Of the 36 plants that had received 450 mg. of phosphorus each week for 4 weeks prior to inoculation, only 28 showed systemic infection within 30 days after inoculation. The symptoms developed on these 28 plants within an average of about 18 days. With the addition of medium amounts of phosphorus (60 mg. each week), an average of 19.4 days elapsed before the appearance of systemic infection. It is evident, therefore, that the addition of medium or high amounts of phosphorus interfered in some way with systemic spread of the virus through the host or the subsequent development of systemic symptoms.

INFLUENCE OF POTASSIUM NUTRITION ON SYSTEMIC INFECTION

The potassium series was carried out during October, November, and December of 1935. The compositions of the nutrient solutions used in this series are given in table I. Each of these solutions contained 20 mg. of nitrogen and 20 mg. of phosphorus per 100 cc. The medium-potassium solution contained 25 mg. of potassium, and the high-potassium solution 500 mg. per 100 cc. of solution. Each of the 7 groups contained 36 plants. Twenty-five representative plants in each group were inoculated as previously described. In table IV are recorded the experimental data derived

TABLE IV

EFFECT OF POTASSIUM ON SYSTEMIC SPREAD OF A YELLOW STRAIN OF TOBACCO-MOSAIC VIRUS

Potassium added each week (mg./pot)					Growth records at time of inoculation*		Percentage of plants showing systemic infection	Number of days elapsed before appearance of systemic symptoms
1st week	2nd week	3rd week	4th week	5th week	Green weight	Height		
mg.	*mg.*	*mg.*	*mg.*	*mg.*	*gm.*	*cm.*	*%*	*days*
0	0	0	0	0	19.4†	20†	100	9.36‡ ± 0.69§
0	0	0	0	1500	19.4	20	100	8.48 ± 0.56
0	0	0	1500	1500	20.3	20	100	14.80 ± 1.09
0	0	1500	1500	1500	19.4	18	100	17.88 ± 1.26
0	1500	1500	1500	1500	18.5	18	100	19.96 ± 1.20
1500	1500	1500	1500	1500	15.7	14	100	18.08 ± 0.89
75	75	75	75	75	29.1	30	100	13.68 ± 1.05

* Plants inoculated at end of 4th week.
† Average of 8 plants in each group.
‡ Average of 25 plants in each group.
§ Standard error of the mean.

from the potassium series. The retardation in growth resulting from the addition of the high-potassium solution for 3 or 4 weeks was slightly more than that brought about by the addition of the potassium-deficient solution. The potassium-deficient plants showed symptoms of potassium deficiency in their lowermost leaves, whereas the high-potassium plants showed no signs of injury except the retardation in growth. All of the inoculated plants showed symptoms of systemic infection within 30 days. In plants grown with the potassium-deficient solution, symptoms of systemic infection appeared within an average of 10 days. The addition of high potassium at time of inoculation further shortened the average time required for the appearance of systemic infection by about 1 day. When high potassium was added 1 week prior to inoculation, symptoms appeared within about 15 days after inoculation. When the high-potassium solution was added for 2 or more weeks before inoculation, symptoms appeared within 18 or 20 days. It is apparent that medium or high amounts of potassium retarded the development of symptoms of systemic infection.

Discussion

The experimental data indicate that the rapidity with which symptoms of systemic infection develop is not governed primarily by the distance the virus must travel to reach the growing tip. The high-nitrogen plants were much larger than the nitrogen-deficient plants, yet systemic infection appeared in both groups at about the same time. Furthermore, the plants which had received high potassium were about four-fifths the size of those

which had received no potassium, yet systemic infection appeared 9 days later in the high-potassium plants than it did in the potassium-deficient plants.

In all tests, symptoms of systemic infection appeared earlier in plants deficient in nitrogen, phosphorus, or potassium than in plants supplied with the element in question. This difference in the time required for the appearance of systemic infection was most noticeable in the phosphorus and potassium experiments. This variation in time reaction may be due to a physiological process which is probably similar in plants deficient in nitrogen, phosphorus, or potassium. In plants deficient in any of these 3 elements, the element in question is translocated from older mature leaves up to the growing tip. When mature leaves of deficient plants are inoculated, the virus may be translocated from these leaves by this re-utilization process faster than it might be translocated from leaves of a similar age on normal plants. The more rapid development of symptoms of systemic infection brought about by the addition, at time of inoculation, of the element previously deficient does not invalidate this explanation. Several days may elapse before the plant resumes normal growth. Meanwhile, translocation from the mature leaves may still be taking place.

There are several factors which must be considered in an attempt to explain the differences in the time required for the development of symptoms of systemic infection in plants receiving various nutrient solutions. Of these factors, the following are the most important: susceptibility of the inoculated leaf, incubation period of the virus, rate of virus multiplication, rate of movement from the inoculated leaf, rate of movement in the stem, and susceptibility of tissue in the tip. In regard to the first factor, it has previously been shown (**3, 4**) that plants receiving various nutrient solutions differ in their susceptibility to primary infection. Because of this variation in susceptibility, all plants were so heavily inoculated that many primary lesions developed on all plants. This heavy inoculation was believed sufficient to offset any effect which variations in susceptibility of the inoculated leaf might have on the subsequent development of systemic infection. The experimental evidence does not indicate what other factors might have been affected by host nutrition. The evidence does indicate, however, that nutrition has produced some change in the disease complex, inasmuch as the time required for the systemic development of the disease varied with the nutrient treatment.

It is not yet known how host nutrition affects the systemic spread of the virus. Mineral nutrition may change the rate of protoplasmic streaming, the nature of protoplasmic connections between cells, or the rate of translocation of food materials. Any changes such as these would probably alter the rate of virus spread within the plant.

Summary

The influence of mineral nutrition on the systemic spread of a yellow strain of tobacco-mosaic virus in tobacco plants in sand cultures was studied by determining the time required for the virus to reach and produce symptoms of secondary infection on tip leaves of inoculated plants.

Symptoms of systemic infection appeared earlier in plants that had received nutrient solutions containing either a deficiency or an excess of nitrogen than in plants that had received a medium amount of nitrogen. It seemed to be immaterial whether the treatment with excess nitrogen started 1 week or 4 weeks prior to time of inoculation.

Plants that had received no phosphorus showed symptoms of systemic infection earlier than plants that had received excess phosphorus for 1 week prior to time of inoculation, and these in turn showed symptoms earlier than plants that had received excess phosphorus for 2, 3, or 4 weeks prior to inoculation. The growth made by the plants was directly correlated with the duration of the excess-phosphorus treatment.

The time of appearance of systemic symptoms was also directly correlated with the duration of the treatment with excess potassium. However, in contrast to the findings in the phosphorus study, the high-potassium treatment retarded growth more than the potassium-deficient treatment.

It is concluded that the systemic development of the disease was accelerated by high-nitrogen nutrition and retarded by either high-phosphorus or high-potassium nutrition. The rapidity with which symptoms of systemic infection developed showed no apparent correlation with the distance the virus had to travel to reach the growing tip.

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ABSORPTION SPECTRA OF SINGLE CHLOROPLASTS IN LIVING CELLS IN THE REGION FROM 664 mμ TO 704 mμ[1]

V. M. Albers and H. V. Knorr

(WITH TEN FIGURES)

Introduction

The absorption of light in living plant material, either in the form of living leaves or suspensions of algae, is a complicated optical process. The problems involved have been treated in detail by Mestre (3). It is well known that the position of the red absorption band of the living material is displaced toward the red end of the spectrum about 20 mμ from that of the red absorption band of pure chlorophyll *a*. Wlodek (5) has suggested that the displacement may be due to an optical property of the living leaf or that it may be due to unstable compounds formed by the pigments in the photosynthetic process.

An attempt has been made by Baas-Becking and Ross (2) to eliminate the complicated scattering processes, usually encountered, by photographing the absorption spectrum of a single *Euglena*, using a microspectrograph. Since they show only a densitometer curve of the spectrogram, and since the resolving power of their experimental arrangement was very low, their results do not give a complete answer to the problem. Furthermore, the *Euglena* cell does not represent a single chloroplast.

This investigation was undertaken for the purpose of determining the absorption spectra of individual chloroplasts, in the region of the red absorption band, with a high resolving power spectrograph, using calibrated plates so that the actual absorption curve in that region could be determined.

Experimentation

The optical system used for photographing the absorption spectra is shown diagramatically in figure 1. It consists of a microscope, with pro-

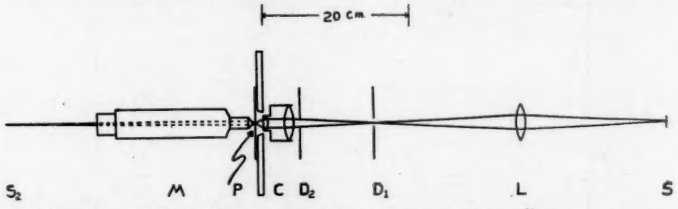

Fig. 1. Optical system used for photographing absorption spectra.

[1] A contribution of the C. F. Kettering Foundation for the study of chlorophyll and photosynthesis.

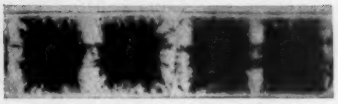

FIG. 2. Photomicrograph of chloroplasts in *Zygnema*.

vision for critical illumination, and a Hilger E_3 spectrograph for photographing the spectra. The chloroplasts are mounted, either in water or water containing some agar, between a glass coverslip and a glass object slide. This preparation is mounted on the stage of the microscope so that the chloroplasts are in the plane, P. The source of light, S, is a six-volt, 108-watt, ribbon filament lamp. The lens, L, forms a real image of the filament at the diaphragm, D_1, which is imaged in the plane, P, by the microscope

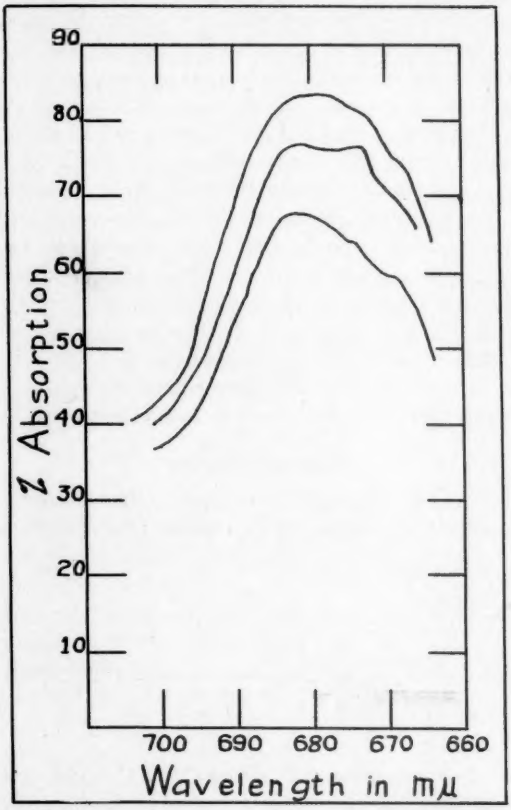

FIG. 3. Absorption spectra of *Protococcus* chloroplasts from one source.

substage condenser, C. The diaphragm, D_2, is a normal substage condenser diaphragm. With this arrangement, D_1 serves as a field diaphragm, while D_2 serves as an aperture diaphragm. The objective used in a 4 mm. achromat of 0.85 N. A. The microscope is so focused that an image of the chloroplast is formed by the objective on the slit of the spectrograph, S_2. No ocular is used on the microscope. The diagram (fig. 1) is drawn to scale in all respects except for the distance from the top of the microscope tube to the spectrograph slit. This distance was 50 cm., giving a magnification of 177 diameters. Figure 2 is a photomicrograph of the chloroplasts in a *Zygnema* filament made with a magnification of 200 diameters. The maximum angular aperture of the cone of light from the preparation which can be collected by an objective of 0.85 N. A. is 116°. In this work the aperture diaphragm of the condenser was stopped down considerably below this value, but no means were available for determining the actual angle of the cone used. The field diaphragm, D_2, is a fixed diaphragm and its diameter is such that a field of

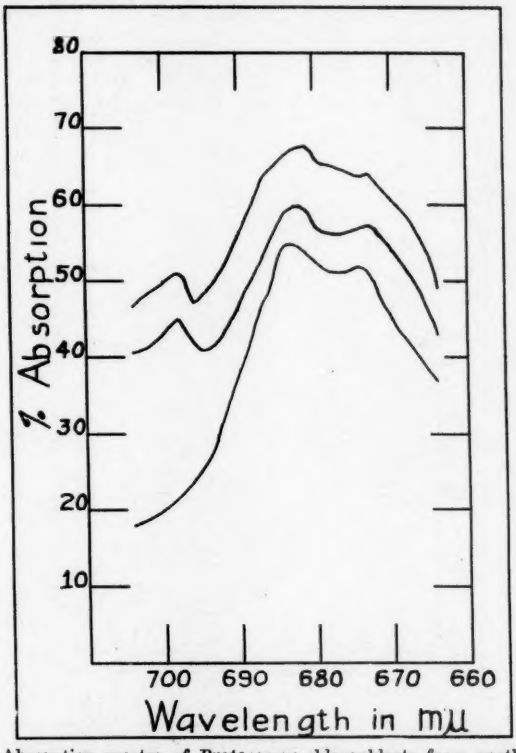

FIG. 4. Absorption spectra of *Protococcus* chlorophlasts from another source.

about four times the diameter of the chloroplast is illuminated. Under these conditions, very little light, not actually transmitted by the chloroplast, can be scattered into the spectrograph slit.

In order to determine the percentage of absorption by the chloroplasts as a function of the wavelength, each plate was calibrated. This was done by moving the slide, on the stage, until the image of the chloroplast did not fall on the slit and making a series of exposures on the plate with oscillating neutral screens of known transmission in the light path. The percentage transmission of the chloroplasts at each wavelength was then determined by the method which has been previously described (1). It is not possible to make any correction for reflections occurring at the first surface of the chloroplast.

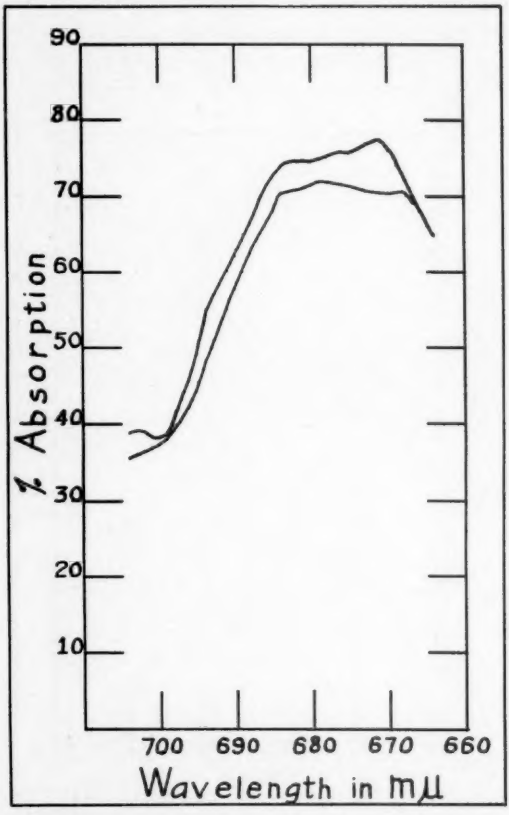

FIG. 5. Absorption spectra of *Protococcus* chloroplasts at a different season.

The sources of chloroplasts used were the algae: *Protococcus*, *Spirogyra*, and *Zygnema*. The spectra were studied only in the region of the red band, *i.e.*, 664 mμ to 704 mμ.

Results

Figures 3 to 6 show the absorption spectra of *Protococcus* chloroplasts. Each absorption curve was made from a different cell. The curves in figure 3 were made from cells gathered from one source and those in figure 4 were made from cells gathered from another source. The two sets of cells were gathered at the same time. The curves in figures 5 and 6 were made from cells which were gathered at the same time and place. They were gathered at a different time of year than those from which the curves in figures 3

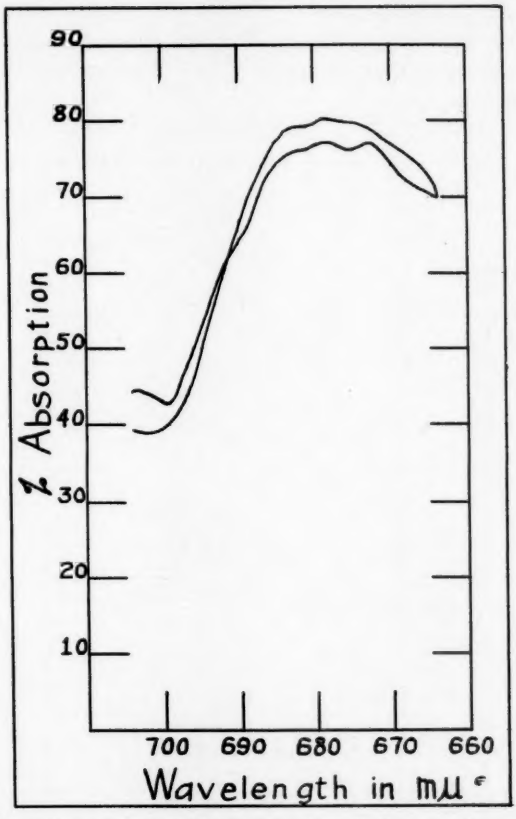

FIG. 6. Absorption spectra of *Protococcus* chloroplasts, same time and place as in figure 5.

and 4 were made. Figures 7, 8, and 9 show absorption curves of the chloroplasts of *Spirogyra*. All of the cells used were gathered from the same source and at the same time. Figure 10 shows absorption curves of the chloroplasts of *Zygnema*. In this case, the chloroplasts were all from cells on the same filament. In all cases, the algae were gathered immediately before the spectra were photographed to insure a minimum of danger of injury to the cells before they were used.

The striking characteristic of these absorption curves is the presence in nearly all cases of several maxima of absorption, rather than a single maximum at about 680 mμ, as ordinarily reported. The relative intensities of these maxima vary from one cell to another, indicating that they are produced by different substances. Table I gives the wavelength readings of the maxima from all of the curves.

In the case of the *Zygnema* chloroplasts, the individual maxima are not as well resolved, although the shapes of the curves indicate that the same bands are present.

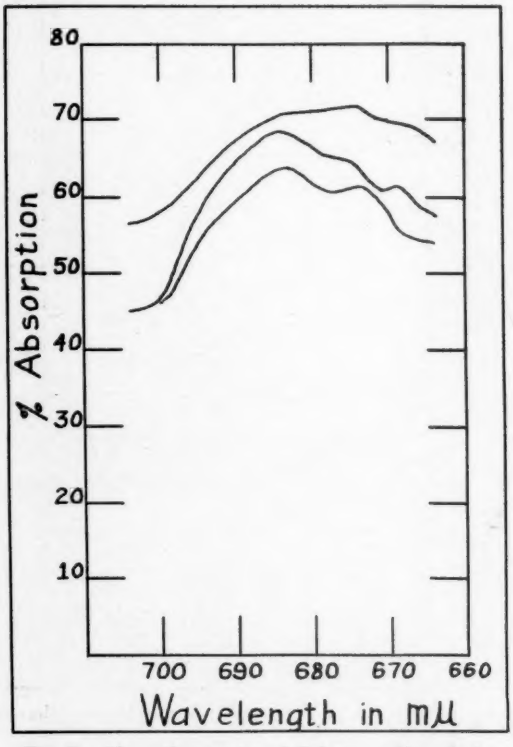

FIG. 7. Absorption spectra of *Spirogyra* chloroplasts.

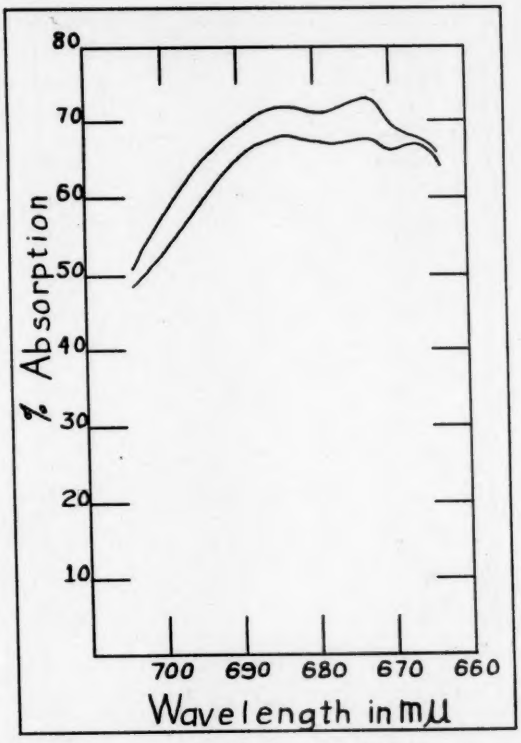

FIG. 8. Absorption spectra of *Spirogyra* chloroplasts, same time and place as in figure 7.

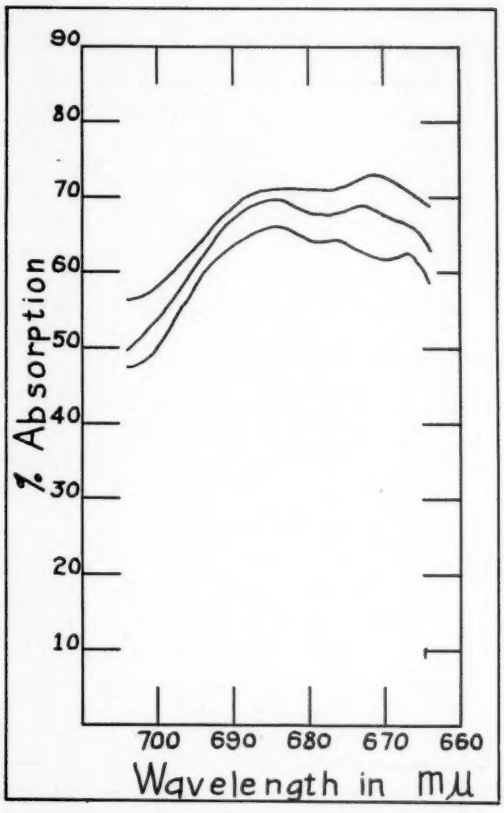

FIG. 9. Absorption spectra of *Spirogyra* chloroplasts, same time and place as in figure 7.

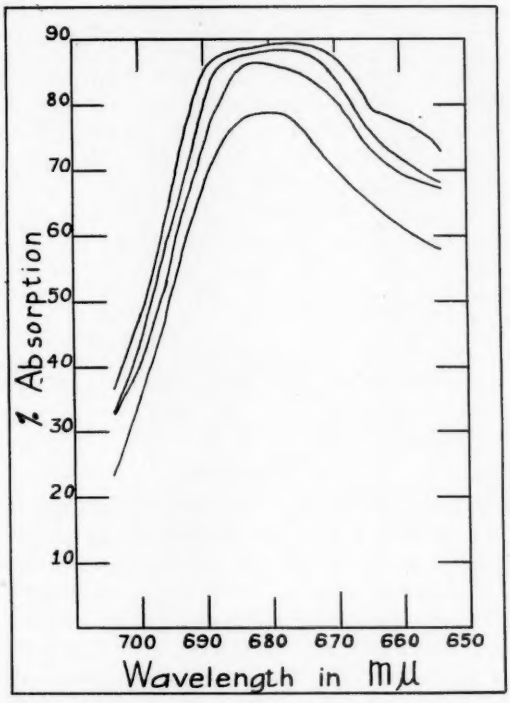

FIG. 10. Absorption spectra of *zygnema* chloroplasts, all from cells of the same filament.

TABLE I

Species of algae	Wavelengths of bands in mμ					
Protococcus (1)	698	687	681		673	
Protococcus (2)	698		682		673	
Protococcus (3)		687	683		674	
Protococcus (4)			←680→		673	669
Protococcus (5)			682		674	
Protococcus (6)			682		674	669
Protococcus (7)			684	679		
Protococcus (8)			683	678	673	
Protococcus (9)			683	676	671	
Protococcus (10)			684	679		668
Spirogyra (1)			684		673	
Spirogyra (2)			684		673	667
Spirogyra (3)			684		676	667
Spirogyra (4)			684		673	667
Spirogyra (5)			682		672	
Spirogyra (6)			683		674	
Spirogyra (7)			684		675	669
Spirogyra (8)			683		674	
Zygnema (1)			←680→			
Zygnema (2)			683		675	
Zygnema (3)		←688→			675	
Zygnema (4)		←687→			675	

Discussion

The readings of the positions of maxima on these curves probably do not, in all cases, represent the positions of the maxima of the individual bands. The bands occur with so little wavelength separation, compared to their width, that one maximum on a curve may be the resultant of two adjacent bands, and the wavelength, read for the resultant, would then be influenced by the relative intensities of the two bands. This is probably true both in the case of the maximum appearing at about 673 mμ and the one at about 683 mμ.

In the spectrum of solutions of the two green plant pigments, chlorophylls α and β, each has a single sharp maximum at 661 mμ and 643 mμ respectively. The band in living leaves occurring at 680 mμ has always been attributed to the chlorophyll α in the leaf. The results of this investigation indicate that there are either other pigments present, giving absorption comparable to that due to chlorophyll α, or that chlorophyll α enters into the photochemical reactions in photosynthesis in several steps, forming reaction-products having absorption bands at slightly different wavelengths. This last assumption seems more reasonable than the first, since one would expect that other pigments, having absorption comparable to that of chloro-

phyll α, would have been isolated by chemists working with the leaf pigments. These results also indicate quite definitely that the wavelength difference between the absorption band, as observed in pure chlorophyll α solutions and the living leaf, is not produced by the optical properties of the leaf tissue.

WILLSTÄTTER and STOLL (**4**) have pointed out that the increased light path in the albino leaf results in an increase in absorption by its tissues, giving a false notion of the actual amount of absorption by the pigments. By the same argument, it is evident that, in a given leaf, the increased path of the light of wavelengths where the absorption of the pigments is less, will result in a corresponding increase in the absorption in the tissues for these wavelengths, thus tending to smooth out the absorption curves. This might explain the fact that all observers working with the entire leaf have observed only a single broad absorption band at 680 mμ instead of several maxima. This idea is still further supported by the fact that the *Zygnema* chloroplasts, which were the largest chloroplasts studied, show the least resolution of the band maxima.

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ON THE DETERMINATION OF PLANT ENZYMES[1]

Z. I. KERTESZ

The fact that plant life depends a great deal on enzymes was recognized early, and enzymes are receiving the constant attention of workers in botanical sciences. Because one deals with enzyme *action* rather than with isolated enzymes, enzyme chemistry is chiefly a science of methods. The results obtained in any specific case may yield a basis for conflicting conclusions. The reason for this lies in the fact that most methods for the estimation of enzymes have been worked out by enzyme chemists rather than by those interested in enzyme chemistry as a tool for the botanical sciences. The methods to be used, and the facts observed will greatly depend on the point of view of the worker. In too many cases, results and conclusions obtained by the botanist by the use of strictly enzyme chemical methods fail to take into account the actual rôle and significance of the enzyme in the plant. The enzyme chemist is aware of the unstable character of the enzyme. The botanist has to go one step farther because he is dealing with the equally unstable and complex plants which produce and contain the enzyme.

From the standpoint of the determination of plant enzymes, the conditions under which the substrates of enzyme action exist in the cell should be considered first. The protoplasm is a constantly changing system in which the different materials exert great influence on each other. It has also been shown that many important chemical constituents of the cell such as proteins, polysaccharides, and lipids may form homogeneous or heterogeneous complexes or aggregates which have different properties, hence behave entirely differently toward enzymes. The size and complexity of such aggregates, furthermore, are undergoing almost steady changes in the protoplasm. Naturally the substrate may also react with other chemical constituents of the cell, producing reversible or irreversible compounds of different affinity to the enzyme. Thus the condition of the substrate of enzyme action in the cell may be entirely different from that in a test-tube, and also may show great qualitative and quantitative variation within a short period of time.

Similar to an enzyme, a substrate may also go through changes making it unavailable or "inactive." The substrate may be separated from the enzyme by occlusion. Occlusion, however, should be regarded as a case of spatial

[1] Read before the joint session of the American Society of Plant Physiologists, American Society for Horticultural Science, and the Physiological Section of the Botanical Society of America with the A. A. A. S., held at Rochester, New York, June 17, 1936. Also an introduction to the recommendations on the determination of plant enzymes contemplated by the Committee on Chemical Methods of the American Society of Plant Physiologists.

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separation. The substrate may be separated from the enzyme by adsorption or by membranes within the cell. Because of its great importance, spatial separation will be discussed in detail later in this paper.

But let us turn to the enzymes and consider the factors which may influence their activity in the cell. We know little about the mechanism of enzyme formation. In many cases, especially in microorganisms, the cell is capable of producing new enzymes to suit changed living conditions; and it is well known that the activity or quantity of the enzyme can be greatly influenced. The much debated existence of "zymogenes" or "proenzymes" is of historical interest only. It has been shown for practically every case that where the presence of a zymogene was contemplated, upon changing the conditions of determination and especially upon the addition of specific activators, the full activity of the enzyme could be attained. Thus the predecessor of the active enzyme is not a zymogene in the old sense of the word but the enzyme in an insoluble form (amylases), or lacking certain chemical changes (or activators) to become active (papain). Indeed, sometimes enzymes are formed from inactive compounds by heat or by the action of other enzymes (**5**).

A very interesting case of this sort is the germination of seeds. It is most probable that in the first stages of germination there are no new enzymes formed or set free from a "zymogene." The increased enzyme activity seems to be caused by the liberation of strongly adsorbed enzymes. The proteolytic activity in the seed commences as soon as the water is taken up. The liberation of the amylases, on the other hand, is apparently the result of the action of proteolytic enzymes. CHRZASZCZ and JANICKI (**2**) established the fact that when dormant wheat, barley, rye, and other cereal seeds are treated with proteolytic enzymes, there is a great increase in the amount of amylase which passes into solution. The amylase was present in the dormant cell with all three functions (liquefication, dextrinization, saccharification) but is inactivated by being adsorbed on a sisto-material (**3**). Later during the germination, however, there is a demonstrable formation of new enzymes. This observation shows how critical and careful one must be with the results of enzyme determinations, and in accepting information available in the literature.

Thus enzymes may be present in plants without exerting any activity. Starch is present in plants containing active amylases but is not hydrolyzed while the cell structure is intact. The presence of inactive enzymes in the cell, apparently very near to the substrates of their action, is another example of the marvelous coordination within the cell.

The inactivity of the enzyme in the cell may be caused by the absence of activators, spatial separation, formation of inactive compounds, and perhaps by a number of other factors. Undoubtedly the case of spatial separation is the most frequent factor and of most importance in controlling enzyme

action *in vivo*. There are two classes of conditions which may cause inactivity of the enzymes by spatial separation. One is occlusion, or a selective adsorption of the enzyme or the substrate on other cell constituents. In this class also belong the cases in which both the enzyme and substrate are adsorbed by the same material, and the enzyme exerts no activity. The complexity of enzyme relations in the cell is well indicated by the observation that in certain cases, when the enzyme and its substrate are adsorbed, the velocity of the enzyme reaction is not altered at all, while in other cases it is completely nullified. Yeast saccharase loses none of its activity when adsorbed by a variety of solids (**8, 9**); on the other hand, amylase is inactive while adsorbed (by the "sisto-material") in ripening and dormant cereals (**3**). Our knowledge of surface reactions must make further advances before we will be justified in doing much speculation on what the mechanism of such reactions is.

The other and probably more common example of spatial separation is that in which the enzyme and substrate are separated by gel-like or other colloidal membranes. Such membranes have been described many times and appear to be common in the cell as one of the chief means of maintaining the organization therein. It has also been observed that protoplasmic granules are often surrounded by a lipoid or fatty film which protects them from the action of proteolytic enzymes. Often upon removal of the fat or lipoid the granules are readily digested by the enzymes (**1**). In the presence of such colloidal membranes the velocity of the reaction will naturally depend on diffusion, the rate of which will be determined by the permeability of the membrane. Undoubtedly selective permeability plays an important rôle in such cases.

Naturally a combination of both classes of conditions may also be the reason for the inactivity of the enzyme in the tissue. With our present knowledge it is often impossible to say whether the inactivity is caused by adsorption or by the separation of the enzyme and its substrates by membranes.

To obtain enzyme solutions from plants, the natural structure of the cell must be destroyed. It was observed long ago that enzyme action in the cell is quite different from the action of the same enzyme after injury or destruction of the cell structure. The action of enzymes in the protoplasm is regulated by different means; but this coordination characteristic of the living cell is disturbed by injury. It is well known that upon destruction of the cell structure by drying, freezing, or by exposing it to the effect of antiseptics the enzyme activity in the plant tissue increases as a result of the disorganization of the cell. This is mostly caused by the fact that, by the destruction of the cell structure, the enzyme gets into more intimate contact with its substrate. Thus by a destruction of the gel-like membranes in the protoplasm,

the rate of the reaction will no longer depend on the rate of diffusion. Similarly other cases of spatial separation are terminated by a destruction of the cell structure.

There are other changes which may come about when the contents of a cell or cells are mixed. It has been shown that in such cases enzymes normally present in the cell, but not in contact, may exert considerable digesting effect upon each other. We have no evidence that all enzymes are proteins but it is safe to say that many of them are. These enzyme proteins may be digested by proteolytic enzymes, causing loss and even nullification of the enzyme activity. This often happens during autolysis of plant extracts.

It was observed some twenty years ago that hydrogen sulphide activates papain. Later work revealed that compounds containing the – SH group, as certain thiol compounds and glutathion, have very beneficial influence on enzyme action. The apparent rôle of glutathion is the reversal of partly oxidized papain into the reduced form (**4**). We do not have any information regarding the necessity of glutathion for the action of papain in the cell. On the other hand all papain preparations can be activated by glutathion, showing that upon mixing the cell contents, or during preparation, a certain reversible oxidation of the enzyme occurs. Strongly reducing thiol compounds, as glutathion, are not rare in nature. Occasionally when the cell structure is destroyed, these compounds find access to certain enzymes and activate them. Many cases of activation by chemical constituents of the cell are also known. It is most likely that, in many cases, natural constituents of the cell have marked influence on the activities of certain enzymes. This appears to be again one way by which the coordination of enzyme action in the protoplasm is governed. Our knowledge of enzyme activators is rapidly increasing (**11**) and much work is being conducted to explain this interesting phenomenon.

One basic difference between plant chemistry and the study of plant enzymes is that for enzyme work the plant material cannot be preserved. The determinations of plant enzymes must be performed on fresh material because the extremely sensitive and labile enzymes are much influenced or even destroyed by drying, heat, alcohol, and other physical and chemical treatments. The only known method to preserve plant material for enzyme work is to freeze it at very low temperatures, and store it at those temperatures, under conditions preventing desiccation. This method is far from being entirely safe, but can be used in emergency and for short periods of time.

The enzyme in plant materials is often determined in the press sap or in extracts. Because of the limited solubility of many enzymes, much care should be exercised in such cases to obtain reliable results. WILLSTÄTTER, for instance, found eight individual amylases in leucocytes, but only four

of them were easily soluble in water; the others appeared to be bound to cell constituents (**12**). Another interesting case in this line is that of zymase. Although LEBEDEW and others long ago described the preparation of cell-free extracts from yeasts, which were able to produce alcoholic fermentation, many workers failed to obtain such active extracts. It was not until recently that NILSSON and ALM (**10**), in VON EULER'S laboratory, made a study of this problem and established the conditions by which the zymase, bound to other cell constituents, can be rendered water soluble. Thus by making an enzymatic analysis of the extract from plants, one is hardly justified in drawing conclusions about the presence and activity of the enzymes in the cell. Attention is called again to the fact that, for a successful extraction of the enzymes, the structure of the cell has to be destroyed by drying, freezing, autolysis, or chemical means all of which may alter the activity of the enzyme for reasons outlined previously. In many cases, working with suspensions of the ground tissue is satisfactory if the enzyme is not easily soluble in water or glycerol.

As far as the quantitative determination of the enzyme activity in the cell sap is concerned, in certain cases conclusions may be drawn from such results as regards the activity of the enzyme *in vivo*. The sap should be expressed at low temperatures, and with pressures not over 6000–8000 atmospheres because higher pressures cause partial or complete inactivation of the enzymes (**7**). The limit of safe pressure has been found to be astonishingly similar for different enzymes.

After having an enzyme extract or a tissue suspension ready for the determination of its enzyme content, a number of questions arise. Should the determination be performed at the natural hydrogen ion concentration of the plant, or at that of the cell sap (if known), or should it be adjusted to the optimum of the enzyme action? What concentrations should be used? What should be done about activation or removal of natural inhibitors? What about temperature, antiseptics, etc.? With the raising of all these questions we arrive at the main point in the present discussion.

The botanist needs the help of the enzyme chemist. Every one of the above questions has to be answered. The answer in most cases cannot be generalized because the factors influencing different enzymes and the conditions insuring their maximum activity vary. It is the function of the enzyme chemist to establish these facts. Having this essential information available, the botanist should proceed to determine under optimal conditions the activity of the enzyme present. Then he should proceed to obtain information on the rôle and significance of the enzyme in the plant. He ought to investigate the enzyme action under as nearly natural conditions as possible.

The most satisfactory way of studying enzymes would be to observe their action in the undisturbed cell. While there is some hope that this may be

accomplished in the future, there are only a few attempts recorded in the literature reporting even moderate success. Ideal experimental conditions for the determination of plant enzymes would be reached by the addition of an infinitesimal quantity of the substrate to the cell, followed by an immediate determination of the enzyme action during an extremely short period of time. The difficulties in performing such a determination are obvious, and we are restricted at the present to an estimation of the quantity of the enzyme present in the tissue. The best approach to ideal conditions suggested thus far for this purpose is the enzymatic microtechnic originated by LINDERSTRØM-LANG and coworkers (**6**). This method is suitable for the determination of enzymes in minute quantities of tissues or extracts and much valuable information has been obtained already on the distribution of enzymes in animal and plant tissues. But here again we obtain information on the possible maximum activity of the enzyme only, and one should exercise great care in drawing conclusions in regard to the actual normal activity of the enzyme in the cell.

Unfortunate as it is, we do not have any dependable method for measuring enzyme activity *in vivo*. The analysis of plant material for different substrates and reaction products of enzyme action by micro-methods is of use sometimes, but the lability of the composition of the cell, and the many different reactions proceeding may entirely mask the changes in the substrate or the appearance of reaction products of the investigated enzyme reaction.

In the foregoing discussion an attempt has been made to show some of the vital points which have to be considered in connection with the determination of plant enzymes. With the application of the methods of physical chemistry, our knowledge of the protoplasm is rapidly increasing; but still more progress is needed before we can be certain of the condition of the enzymes in the cell. Our knowledge of enzymes, on the other hand, is advancing rapidly, and we can be hopeful of important gains in the not too distant future. While we know enough of the factors which have to be considered in connection with enzyme studies on plants, yet we are not able to make studies on the plant cell in its natural condition. Realizing the need for such methods is the first step toward attaining them.

Meanwhile it is of utmost importance that all work on plant enzymes be made by dependable methods, having clearly in mind all known factors which do or may influence enzyme action. But of even more importance is it that great care should be exercised in drawing conclusions from results obtained *in vitro* regarding the natural condition, activity, and rôle of enzymes within the plant cell.

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INCREASE OF TRANSPIRATION RATES OF TOMATO LEAVES DUE TO COPPER SPRAYS[1]

KENNETH K. KRAUSCHE AND BASIL E. GILBERT

Several observers have reported that leaf transpiration is increased with many plants by the presence of copper sprays. It has also been quite definitely proven, especially by WILSON and RUNNELS (**4**) with *Coleus,* that the greatest increase takes place at night. Little data, however, have been gathered to explain this phenomenon. This paper reports certain experiments carried out with tomato plants which both substantiate the findings of earlier workers as to the increase of transpiration rates due to copper sprays and give some evidence as to the mechanism by which it takes place.

I. Effect of copper sprays on transpiration

Tomato seedlings were transplanted to 5-inch flower pots which had been made impervious to moisture by treatment with paraffin. Each pot contained the same weight of soil of known moisture content.

When the plants had reached a height of 7 to 10 inches, a disc of waterproofed paper, with a hole in the center for the stem of the plant, was paraffined over the top of the pot. Cotton was used around the base of the stem as a plug for the hole. The plug was removed when it was necessary to add more water to the soil.

The transpiration rate of each plant during the day and night was determined as follows: The soil moisture was made up to the desired percentage, and the plants were set on a bench in the greenhouse. Weighings were made at 6:30 A.M. and at 6:30 P.M. The loss in weight was the amount of water transpired during the previous 12-hour period. At each weighing, enough water was carefully added through a pipette to bring the weight back to the original.

After the transpiration rates had been established, the plants were paired, each pair made up of two plants showing approximately equal water loss. One plant of each pair was called the test plant (T), and the other the control plant (C). In some cases, three plants were grouped together—each of two test plants being checked against the same control. The ratios T : C for day and for night were calculated. Then the test plants were sprayed at the beginning of the day period with the desired spray, and the control plants were sprayed with water. The transpiration rates of all the plants were observed during that day and during the following night. The day ratios and the night ratios of T : C were again calculated. The ratios T : C after

[1] Published by permission of the Director of Research as Contribution no. 505 of the Rhode Island Agricultural Experiment Station, Kingston, Rhode Island.

spraying were then computed on the basis of making T : C for the corresponding period before spraying equal to unity. The change in ratio gave a measure of the effect of the spray on transpiration. This procedure is similar to that instituted by DUGGAR and COOLEY (2) and used since then by several other investigators.

The Bordeaux formula usually recommended for early blight on tomatoes is 4–4–50, while the strength of Burgundy spray advocated is usually 1.5–1.5–50. It will be noted that the formulae used in this investigation are much stronger than these. They were purposely chosen, after preliminary tests, in order to insure a measurable amount of visual spray injury. The lime used in making the Bordeaux was slaked lime and contained 68 per cent. calcium oxide. Burgundy spray was prepared by mixing 1 pound of copper sulphate and 2 pounds of sodium carbonate in 50 gallons of solution. Household washing soda was used as the sodium carbonate.

Each spray was tested for excess available copper with a 10 per cent. potassium ferrocyanide solution. Twenty cc. of the spray solution were put in a beaker and a few drops of potassium ferrocyanide solution were added. A red-brown precipitate indicated the presence of available copper in the spray. A negative test indicated that all the copper was in a combined and insoluble form. The Bordeaux 8–8–50 gave a negative copper test. The Bordeaux 8–4–50 gave a decidedly positive test and the 8–12–50 a negative test. The Burgundy 8–8–50 contained a great excess of available copper.

In the first series, three trials were made, each using sixteen plants. Four different sprays were employed: Bordeaux 8–8–50, 8–4–50, 8–12–50, and Burgundy 8–8–50. The soil moisture was kept at 15 per cent. The values of the ratio T : C of the sprayed plants (when T : C of the control plants equals 1.00) are tabulated in table I.

TABLE I

EFFECT OF CERTAIN COPPER SPRAYS ON TRANSPIRATION OF POTTED TOMATO PLANTS AS SHOWN BY THE RATIO OF THE TEST PLANTS TO CONTROL PLANTS AFTER SPRAYING THE FORMER

SPRAY USED	T : C AFTER SPRAYING	
	DAY	NIGHT
Bordeaux 8–8–50	0.87	1.52
Bordeaux 8–12–50	0.91	1.54
Bordeaux 8–4–50	0.91	1.72
Burgundy 8–8–50	1.18	2.54

Similar trials were made to determine the effect of the spray film applied to the upper surfaces of the leaves only, and to the lower surfaces only. In applying the spray to a plant, the plant was held in one hand either facing

directly into or directly away from the spray stream as it issued from the nozzle. By careful manipulation, a good coverage could be obtained on either surface alone. The spray used was Bordeaux 8–8–50. The soil was kept at 15 per cent. soil moisture. In table II the results are summarized.

TABLE II

EFFECT OF METHODS OF APPLICATION OF BORDEAUX 8–8–50 ON TRANSPIRATION OF POTTED TOMATO PLANTS AS SHOWN BY THE RATIO OF TEST PLANTS TO CONTROL PLANTS AFTER SPRAYING THE FORMER

LEAVES SPRAYED	T : C AFTER SPRAYING	
	DAY	NIGHT
On both surfaces	0.87	1.52
On upper surface	1.01	1.30
On lower surface	0.92	1.18

Table III shows the results of spraying potted plants growing in saturated soil (30 per cent. soil moisture) compared with the sprayed plants growing in 15 per cent. soil moisture. The spray used was Bordeaux 8–8–50.

TABLE III

EFFECT OF BORDEAUX 8–8–50 ON TRANSPIRATION OF POTTED TOMATO PLANTS WITH DIFFERENT SOIL MOISTURES AS SHOWN BY THE RATIO OF TEST PLANTS TO CONTROL PLANTS AFTER SPRAYING THE FORMER

SOIL MOISTURE	T : C AFTER SPRAYING	
	DAY	NIGHT
%		
15	0.87	1.52
30	0.66	1.86

In all these tables, the data are recorded separately for day and for night because of the increased significance which this method lends to the figures. It should be stated, however, that in all cases the total transpiration for the 24-hour period was increased by spraying, from a few per cent. to 105 per cent. There was considerable variation among the plants receiving the same treatment but the trends were consistent in all cases.

At the conclusion of these runs, some of the plants were allowed to remain on the bench without the addition of water. In every case, the sprayed plants wilted perceptibly before the control plants were affected.

The results of experiments on transpiration agree with those of WILSON and RUNNELS (**4**) and those of other investigators in that they show that spraying with Bordeaux and Burgundy increases the transpiration of tomato plants and that the greatest effect is at night.

The greatest effect was brought about by those sprays which were very likely to cause injury. This is shown by data in table IV which resulted

TABLE IV

AMOUNT OF SPRAY INJURY IN THE FIELD ASSOCIATED WITH DIFFERENT COPPER SPRAYS

GROUP*	PERCENTAGE OF LEAFLETS INJURED			
	BURGUNDY 8–8–50	BORDEAUX 8–4–50	BORDEAUX 8–8–50	BORDEAUX 8–12–50
	%	%	%	%
I	85.0	14.0	1.7	3.5
IV	36.0	0.8	1.0	0.0
III	28.5	0.2	0.0	0.2
VI	4.5	0.0	0.0	0.0
V	7.0	0.8	0.0	0.0
VII	5.2	0.2	0.0	0.0
II	7.5	0.0	0.0	0.0
IX	2.5	0.0	0.0	0.0
VIII	4.3	0.0	0.0	0.0

* These groups illustrate varying climatic conditions.

from a field study of the effect of environmental conditions upon spray injury. Burgundy 8–8–50 produced greater increases in both transpiration rates and spray injury than the other sprays used. It perhaps might be expected that severely injured foliage would lose water more rapidly than foliage only slightly injured. It is thus also possible to conceive of the increased transpiration rate as being associated with spray injury.

II. Microscopical and microchemical investigations

There being little doubt that copper sprays bring about increased transpiration and spray injury under certain conditions, the mechanism of this phenomenon is of great interest. It seemed desirable to study stomatal behavior in order to determine any relationship which may exist with increased transpiration, especially since WILSON and RUNNELS (**4**) have suggested that "the accelerating influence of a Bordeaux film on the transpiration rate of a plant is due chiefly to a change in the rate of cuticular transpiration."

Stomatal counts were made on the upper surface and on the lower surface of the leaves using the Leitz ultrapak in conjunction with a Spencer research microscope. The Leitz ultrapak is an attachment which focuses a beam of light, from an illuminator, on the upper surface of the object at the focal point of the microscope. Thus, the leaves could be observed in their natural state while still attached to the plant.

Potted tomato plants about 10 inches in height were used. Pots were set on their sides and leaves held in place on the stage by strips of paper. Both large and small leaves of several plants were used. A total of 100 fields were counted on the lower surface and an equal number on the upper surfaces. Table V shows the results of the count. There were 61.6 times as many stomata per unit area on the lower surface as on the upper surface.

TABLE V

STOMATAL COUNTS MADE ON THE FOLIAGE OF POTTED TOMATO PLANTS

SURFACE	AVERAGE PER FIELD	AREA OF FIELD	THOUSANDS PER SQUARE INCH
		sq. in.	
Upper	0.123	0.0000873	1.41
Lower	7.580	0.0000873	86.83

Although the number of stomata per unit area on the lower surface was 61.6 times as great as on the upper surface, no difference of such great magnitude existed between the change in transpiration when leaves were sprayed on the lower surface and when they were sprayed on the upper surface. In fact, where the stomata are more numerous, the spray film on the lower surface significantly depressed transpiration during the day.

A series of observations was made with the same apparatus, in order to observe directly the effect of sprays on stomata. Some of the plants were sprayed with Bordeaux 8–8–50, while others were untreated. The spray was applied in the morning and observations were made at 3- to 6-hour intervals for more than 24 hours. During this period the soil moisture of both sprayed and check plants was kept at 15 per cent.

Observations on the sprayed leaves were made either directly through a thin film of spray or by flaking off a particle of spray and immediately observing the uncovered stomata.

In observing the stomata, it was found that their general appearance varied very little at any time. The average stoma appeared elongated, with a narrow slit-like stomatal pore about 15 μ long which never opened very widely, if at all. Occasionally one was found which was shorter and much wider than the rest. If any visible opening existed between the guard cells, the stomata were called open; if the pore was found to be definitely closed, it was recorded as closed. There were many stomata, however, which even after careful and patient study could neither be called definitely open or closed because of a blurred appearance in the stomatal pore or because of other reasons. These were recorded as doubtful. The percentage of the total number of stomata in the several conditions was computed for each observation. The summary of the study appears in table VI.

TABLE VI

CONDITION OF STOMATA OF SPRAYED AND UNSPRAYED POTTED TOMATO PLANTS DIRECTLY OBSERVED WITH A LEITZ ULTRAPAK

TIME	UNSPRAYED			SPRAYED		
	OPEN	CLOSED	DOUBTFUL	OPEN	CLOSED	DOUBTFUL
11:30 A.M.	73.7	12.4	13.9	69.5	9.3	21.2
2:30 P.M.	80.9	9.5	9.6	79.3	6.5	14.2
5:30 P.M.	14.5	85.5	0.0	16.5	74.7	8.8
8:30 P.M.	22.6	71.7	5.7	23.0	72.3	4.7
11:30 P.M.	20.2	69.0	10.8	18.7	69.2	12.1
5:30 A.M.	46.8	42.6	10.6	44.0	42.0	14.0
9:30 A.M.	59.4	26.5	14.1	57.9	26.5	15.6
1:00 P.M.	70.0	27.0	3.0	64.5	30.5	5.0

It is safe to say that the general appearance of the stomata of sprayed tomato leaves and those of the unsprayed tomato leaves were the same, except that some of the stomatal pores of the former appeared to be clogged or sealed by minute particles of dried spray.

Spots of spray injury on the leaves were examined around the margin, where the injury had not entirely destroyed cellular structure, in a search for evidence of a relationship between stomatal behavior and spray burn. The burn appeared to involve the ordinary epidermal cells and stomata indiscriminately.

It was indicated by the results of the preceding work that the effect of copper sprays on tomato plants is not primarily a phenomenon of the stomata. Attention was then focused on the epidermis of the foliage and microchemical tests were made in order to determine its chemical nature.

Two series of tests were performed: one on the leaves of potted tomato plants 1 foot high and grown in the greenhouse; and the other on the foliage of mature plants grown in the field. Freehand sections were made and tested with standard microchemical reagents.

Cross sections of the tomato leaf were placed on a glass slide. A few drops of concentrated sulphuric acid were allowed to run onto a section while it was being observed. The action of the acid on the epidermis was noted.

Other sections were saturated with iodine-potassium iodide reagent and any color changes noted. Then 75 per cent. sulphuric acid was allowed to flow over the section and further color changes were observed.

A third test consisted of wetting fresh sections with a solution of chloriodide of zinc and observing the color reaction in the epidermis.

The last test consisted of placing the sections on a glass slide in contact with a solution of Sudan III. The epidermis was examined for evidence of the absorption of the dye.

The results of the two series were the same. The epidermis was immediately soluble in concentrated sulphuric acid. With the iodine-potassium iodide reagent the epidermis was not colored, but upon the addition of 75 per cent. sulphuric acid the epidermis became a deep blue. With chloriodide of zinc, the epidermis assumed a violet hue. Sudan III did not color the epidermis. From these tests it was concluded that the epidermis was composed of undifferentiated cellulose; that is, cellulose which had not become lignified, cutinized, or suberized.

Since all the phases of the work on stomata led to the same conclusion, that they are not the mechanism through which copper sprays increase transpiration, then the action must take place directly through the epidermis. If the epidermis be composed of undifferentiated cellulose or of lignified cellulose it is permeable, while cutinized and suberized cell walls are not, as indicated by STILES (3). Since it was ascertained that the epidermis of the tomato plants used in the experiments was composed of undifferentiated cellulose, they were permeable. The presence of a permeable epidermis allows a reasonable explanation of the passage of water outward from the leaf, without involving the stomata, by the process of exosmosis caused by the presence of the spray film on the surface.

Conclusions

It is probable that the soluble copper and calcium on a leaf surface, so readily permeable as a celluluose membrane is thought to be, will readily penetrate to bring about changes in permeability in the membranes of the guard and mesophyll cells so that water loss takes place at varying rates, That copper does change the permeability of cell membranes has been shown by COOK (1) with *Nitella*.

From earlier work, substantiated by the experiments outlined in this paper, it seems evident that copper sprays increase transpiration particularly at night.

Furthermore, evidence is given to show that with tomato leaves the major portion of the increased transpiration is not stomatal and that copper sprays do not seem to affect the behavior of stomata.

Transpiration with the tomato must take place largely through the cuticle and this must be subject to great modifications in rate by copper sprays.

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PROOF OF THE FLUX EQUILIBRIUM RELATION[1]

GORDON MARSH

(WITH THREE FIGURES)

Introduction

A quantitative connection has been derived (**3**) between the measured E.M.F. of a living cell or tissue possessing electrical polarity and the velocity of cell oxidation, following LUND'S (**1**) oxidation-reduction theory of bioelectric currents. The equations provide a consistent description of the major facts of continuous bioelectric currents and respiratory exchange. The respiratory process was schematized as follows:

$$X \underset{k_2}{\overset{k_1}{\rightleftarrows}} AH_2 + \underset{k_0}{\overset{O_2}{\uparrow}} \xrightarrow{k_3} H_2O + A \xrightarrow{k_4} y \rightarrow CO_2$$

where X is the precursor of the reductant, AH_2, oxygen is supplied by diffusion from the external medium, and the oxidant, A, is destroyed through further transformation. k_1, etc., are reaction velocity constants, k_0 the diffusion constant for oxygen. At a constant rate of oxygen uptake, the oxygen pressure at a locus (phase boundary or structure behaving as an electrode) within the cell will have a constant or flux equilibrium value, P_c. The electrical polarity of a cell will be determined by at least two oppositely oriented loci and may be expressed as

$$E_p = -RT/2F \ln P_{c_1}/P_{c_2}$$

where the subscript **1** designates the locus of high positive potential as measured externally. The negative sign is conventional only. It was assumed that the velocity constant k_1 is smaller at the locus of high positive potential and that this is the principal difference in the reactions at the two loci. For a tissue, the polarity potentials of the individual cells will sum algebraically, or $E = \Sigma E_p$.

P_c may be expressed as a function of the oxygen pressure of the medium, P, and the velocity constants of the respiratory reactions, so that the relation of E to P can be inferred. The equations predict that (1) E will be zero when P is zero; (2) as P increases E will increase to a maximum, decreasing again to zero as P becomes indefinitely large; (3) the increase and decrease will be reversible. The position of the maximum on the P axis can only be determined empirically.

[1] Aided by a grant from the Rockefeller Foundation for work on cellular physiology.

In the present paper experimental verification of the second and third predictions is presented for the E.M.F. of the onion root tip. The first prediction has already been essentially realized (5).

Apparatus and method

The pressure chamber was a cylinder of 2800-cc. capacity made of one-half inch steel pipe. A cover was held in place by twelve bolts through its circumference and through a flange welded to the top of the cylinder. A milled ring on the cover sealed against an asbestos gasket on the flange. A wooden frame held the chamber steady when the bolts were tightened. The cover was provided with an escape valve, a pressure gage reading to 1000 lb. per square inch and a safety valve set to release at 1000 lb. The cover could be tightened to retain 900 lb. for several hours, although as a matter of convenience it was usually permitted to leak slowly. Above 900 lb. there was leakage through the safety valve. The source of pressure was a standard oxygen cylinder connected to the chamber by copper tubing. Water-pumped nitrogen (stated to be 99.8 per cent. pure) was obtained in a cylinder fitting an oxygen needle valve.

The onion bulb was held in a spring clamp to the support of a rack and pinion stand and was wrapped in a sheet of rubber dam to provide insulation and to protect against injury. The root tip was immersed to about 1 mm. in the fluid of the apical electrode cup. The latter was a 65-mm. glass funnel whose shape minimized any rise in fluid which might accompany the solution of large quantities of gas. The basal electrode cup made contact with some region of the root 10 to 20 mm. from the tip, by means of a glass side-tube ending in a loose-fitting claw. With proper adjustment of the initial height of fluid, a considerable volume change could occur in the cup without affecting the contact. A Petri dish of distilled water was placed in the chamber to provide a moist atmosphere.

The electrodes were Ag : AgCl, sealed with DeKhotinsky cement into small glass tubes, and were frequently recoated with chloride. They gave erratic and drifting potentials in tap water, but were stable in one part Ringer's solution to nine parts tap water, which was used as the fluid in the electrode cups.

The measuring instrument was a Compton quadrant electrometer with one quadrant grounded. The basal electrode lead was soldered to the interior of the chamber and the whole grounded from the outside. The apical electrode made connection with the other quadrant through a copper wire baked into the core of a 14-mm. spark plug[2] screwed into the chamber wall, so that the potential measured was that of the tip with respect to the base.

[2] Two such plugs were provided through the courtesy of the Champion Spark Plug Company of Toledo, Ohio, to whom grateful acknowledgment is made.

The pressure gage was scaled in 20-lb. steps and was read to the nearest 5 lb. The E.M.F. was recorded at 30-second intervals during or immediately following any change in pressure, and at longer intervals as it approached a steady value. The pressure was usually elevated abruptly; decompression was carried out more slowly, to permit exchange of gases with a minimum of disturbance to the root or to the electrode fluids. The roots were examined for injury following each experiment by tests of turgidity, opacity, and subsequent growth. There was no evidence that pressures up to 1000 lb. had any injurious effect.

Results

The effect of oxygen, and the existence of a maximum in the E.M.F. curve, is shown in figure 1. E is the inherent E.M.F., P the oxygen pressure,

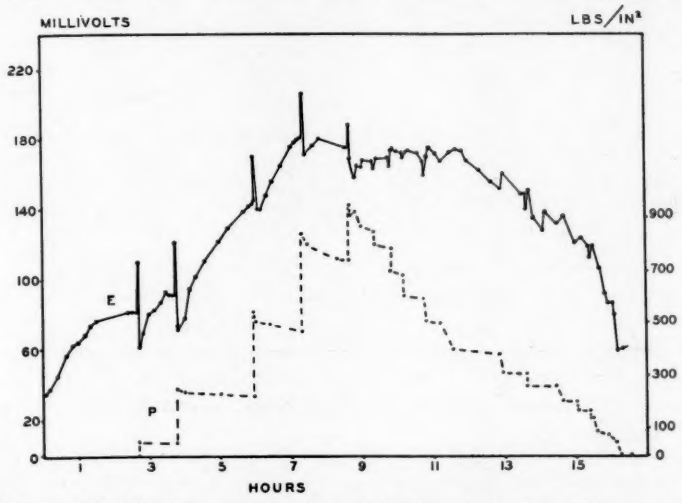

FIG. 1. Potential: time curve for the root at different pressures of oxygen. E, root potential, scale to left; P, oxygen pressure, scale to right. Root tip submerged to 1 mm., basal electrode cup 13 mm. from tip.

plotted against time. In the initial portion of the curve, the root was exposed to air in the chamber. After the E.M.F. had reached a steady value of 82 millivolts, oxygen was admitted to a pressure of 40 lb. The E.M.F. rose instantly to 110 mv., dropped to 62 mv., then gradually climbed to a new steady level of 92 mv. A similar cycle of changes in E.M.F. followed the changes in pressure from 40 to 230 lb. and from 230 to 500 lb. When the pressure was increased to about 800 lb., the final level of E.M.F. was 5 mv. less than that at the lower pressure. Increase of pressure to around 900 lb.

was followed by a further decrease. The pressure was then released in smaller steps. Each abrupt decompression was followed by a cycle of change of E.M.F. roughly the reverse of that produced by an increase of pressure. As the pressure fell from 900 to 600 lb. the E.M.F. rose, to decline again as the pressure was still further reduced. At the end of the experiment, the E.M.F. was about 60 mv. The maximum with increasing pressure lay between 500 and 800 lb.; with decreasing pressure it lay between 800 and 600 lb.

The response of another root to oxygen pressure is shown in figure 2.

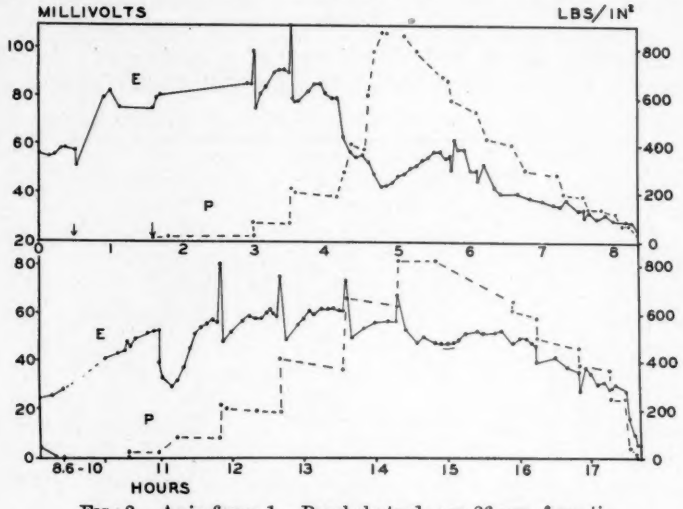

FIG. 2. As in figure 1. Basal electrode cup 26 mm. from tip.

For one hour and five minutes (between the arrows) oxygen was passed through the chamber with the escape valve open. The valve was then closed and the pressure elevated. The E.M.F. increased with the first two pressure increments; but when the pressure was raised from 75 to 200 lb. it dropped 10 mv. and continued to fall as the pressure was increased to 400 and to 860 lb. With release of pressure there was a small recovery with a maximum of about 600 lb., then a steady decline until the pressure had nearly reached zero. For two hours the root was left undisturbed in an atmosphere of oxygen, during which time the E.M.F. rose to a steady level some 10 mv. below that at the beginning of the experiment. The pressure was again increased, the maximum E.M.F. now appearing between 350 and 650 lb. Once more, as the pressure was released, the E.M.F. underwent a small but definite recovery, passing through a maximum as the pressure fell below 700 lb. The last three pressures at which the maximum appeared are in rough agreement.

Ten roots showed the phenomenon of a maximum E.M.F. with increasing oxygen pressure. Two other roots gave increasing potentials up to 570 and 520 lb. respectively, the highest pressures employed. Three roots gave erratic results, showing no consistent effect of oxygen pressure. In general the position of the maximum is well marked with increasing pressure, less well so with decreasing pressure. In part this is apparently due to a slower adjustment of the E.M.F. to a steady level with decreasing pressure, as may be seen in the first decompression in figure 2. It was also generally true that the maximum E.M.F. reached on decompression was less than that reached with increasing pressure.

The electrodes alone were repeatedly tested in the chamber with a salt bridge connecting the electrode cups. Their potential did not vary with oxygen (or nitrogen) pressure.

In order to determine whether the above effects were due to oxygen, as such, or to changes in the physical environment associated with alteration of pressure, the potential of four roots were followed with nitrogen at different pressures. Calculation shows that temperature effects from changing pressure-volume or vapor-pressure relations in the chamber are negligible. The pressure, however, might act as a mechanical stimulus; or temporary evaporation of water from, or deposition upon the root surface could conceivably be responsible for all or part of the changes in E.M.F.

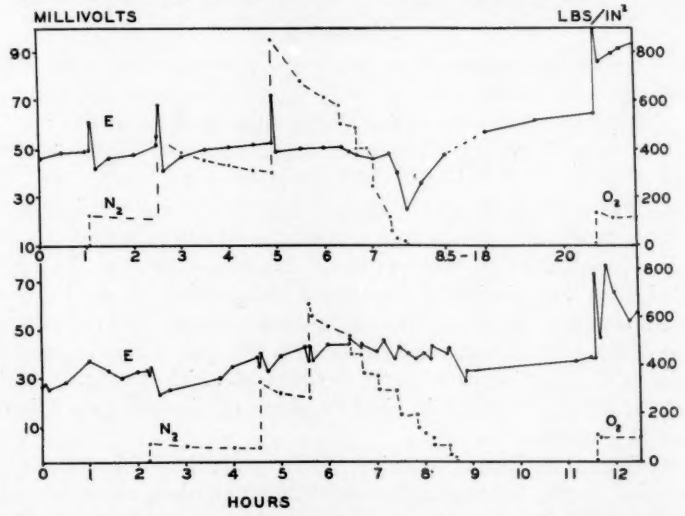

FIG. 3. Effect of nitrogen upon potential of two roots. N_2, nitrogen pressure; O_2, oxygen pressure. In both curves, root-tip submerged to 1 mm., basal cup 10 mm. from tip.

Figure 3 shows two such experiments. At the start the chamber was filled with air. In the upper curve each elevation of nitrogen pressure produced a sharp rise and fall of E.M.F., following which the initial value was approximately reached. No change in E.M.F. occurred on decompression until the last 100 lb. was released, when the potential fell to about half its steady value and recovered. The root remained in the chamber over night, as indicated by the break in the curve. Oxygen was then admitted to 110 lb. pressure, producing a large increase in E.M.F. which was maintained above the level in nitrogen for some two hours beyond the end of the curve.

The lower curve of figure 3 is essentially similar. The small increase in potential which occurred during exposure to nitrogen is probably to be attributed to the normal variation in potential (**2**) since the same rate of increase may be seen when the root was exposed to air. At the end of the experiment, oxygen was passed into the chamber to 100 lb. and the E.M.F. rose from 38 mv. to become steady around 52 mv.

It is evident that pressure alone is not responsible for the changes in the steady level of E.M.F., and that the oxygen pressure relation is a real one. The transition curves from one steady level to another, following an abrupt change in pressure, appear with nitrogen, although their magnitude and character are different from the corresponding curves with oxygen. The difference is particularly striking when obtained upon the same root, as in figure 3.

Discussion

The experiments presented in this paper are concerned more with the demonstration of the existence of a maximum in the E.M.F.-pressure curve than with the precise determination of the pressure at which the maximum appeared. The most serious limitation to that precision lies in the extreme lengths of time involved in completing a single experiment. As may be seen in figures 1 and 2, one to two hours or more are required for the E.M.F. to attain a new steady level following a pressure change. The maximum is apparently not a pressure point, but a pressure range. This is to be expected upon the principle of summation of potentials (**2, 4**), for there is no reason to expect the maximum to be the same for all cells of the root.

The position of the maximum varies from one root to another, and there is evidence of a relation between the pressure at which it appears, and the normal potential of the root in air. Further experiments upon this point are now in progress.

The existence of the maximum is a specific prediction of the equations for the flux equilibrium for respiration and E.M.F., and is a consequence of no other mechanism for the production of potentials in living cells. It may, therefore, be regarded as a proof of the validity of the flux equilibrium concept.

Summary

1. As the oxygen pressure of the environment of the onion root tip is increased, its inherent E.M.F. increases, passes through a maximum, and then decreases.

2. The effect is reversible; *i.e.*, the maximum is found with both increasing and decreasing oxygen pressure.

3. Neither pressure, as such, nor altered vapor tension is responsible for the effect, since the E.M.F. is constant in different pressures of nitrogen.

4. The existence of the maximum fulfills a specific prediction of the equations for the kinetics of an oxidation-reduction system for respiration and E.M.F. at flux equilibrium.

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VALUE OF THE DYE-ADSORPTION TEST FOR PREDETERMINING THE DEGREE OF HARDINESS

STUART DUNN

Introduction

The problem of the determination of cold hardiness of plants by methods other than that of the natural one of survival under field conditions has been the subject of numerous investigations during recent years. The chief purpose of most of this work was to evolve a laboratory test that could be used instead of more uncertain and longer field tests.

The dye-adsorption test, in which hydrophilic colloid content is measured colorimetrically by the amount of a dye, such as malachite green adsorbed from aqueous solution, has been shown to offer some promise in the measurement of such material and has given a strong indication that colloidal constituents are in considerable degree responsible for hardiness (**2**). It was also found that there were great variations in hardiness of individual plants, such as cabbage, in a group chilled simultaneously and that dye adsorption values also varied, but that when a plant first was found to be hardy or nonhardy by a freezing test and then some of its tissue subjected to a dye-adsorption test, the amount of dye adsorbed was found to be correlated with hardiness.

In view of these individual variations and because no hardiness test can be regarded as of practical value if it is not sensitive enough to determine hardiness in advance of cold treatment, it seemed desirable to determine the sensitivity of the dye-adsorption test in that respect.

Experimentation

In the experiments to be reported *Bryophyllum* and cabbage plants were used. *Bryophyllum* plants are easy to grow and they produce a large number of leaves; which factor is important in sampling. At the same time they allow a sufficient number of leaves to remain on the plant to demonstrate injury by freezing. Cabbage plants have much the same qualities and are also rather hardy.

Experiments with *Bryophyllum*

For the first series of determinations a large number of large-sized, vigorous plants were chosen and divided into several groups varying in number from 10 to 16 plants. A composite sample of several leaves from each plant in a given group was taken and macerated and tested for dye adsorption by the usual colorimetric procedure (**2**). One-gram samples were treated with 25 cc. of 0.02 per cent. malachite-green solution. As soon thereafter as pos-

sible all the plants in that group were subjected to a temperature of – 1.1° C. for 15 hours. Previous work had shown this to be the right testing temperature. The degree of injury was noted by expressing the results as percentage of the whole plant uninjured or surviving this temperature treatment and this theoretically relates to the amount of dye adsorbed by the tissue. Seven groups of plants comprising a total of 86 individuals were treated in this way and the results, as summarized in table I, show a wide variation in

TABLE I

COMPARISON OF DYE ADSORPTION VALUES WITH FREEZING RESULTS FOR WHOLE *BRYOPHYLLUM* PLANTS

SURVIVAL	DYE ADSORBED	MEAN	RANGE
%	%		
100	90–88–84–60–60–46–37	66	90–37
95	57–	57	.....
90	75–53	64	75–53
85	70–35	52	70–35
80	58–51	54	58–51
75	55–28–28	37	55–28
50	75–70–59–54–50–44–31	54	75–31
40	51–	51	.....
35	45–35	40	45–35
30	57–	57	.....
25	58–55–45–18	44	58–18
15	70–59–42–35	51	70–35
10	63–57–54–51–42–31–29–28–17	41	63–17
5	72–68–60–55–54–51–39–15	51	72–15
0	78–75–71–70–67–66–66–65–64–	.....	.....
.....	64–62–57–56–55–55–54–53–53–	.....	.....
.....	53–51–50–49–48–48–45–43–38–	.....	.....
.....	36–34–33–29–21	53	78–21

survival, with several plants totally uninjured, a much larger number entirely killed, and a scattering number intermediately injured. This table has been arranged to show the dye adsorption results for each individual plant grouped together on the basis of their percentage of survival and showing the range and mean value of the dye adsorption results for the plants in each survival group. Inspection of these results shows that for the survival groups containing several plants the range between the high- and low-adsorption values is very large, showing some correlation with hardiness for some plants and none at all in others. The mean values for the different survival groups also show no definite trend of agreement with survival.

Sampling from so large a unit as a whole plant gave results so inconclusive, that a series of experiments was undertaken to secure more representative sampling from smaller, more localized portions of the plant. To this end a group of large *Bryophyllum* leaves was selected and detached

from the plants. They were each halved lengthwise on one side of the midrib and the half with the midrib was placed with the petiole-end in a flat of moist sand, to be tested for its resistance to injury by freezing. Again this plant is rather well adapted to this form of test. Because of its heavily cutinized epidermis it does not wilt readily under these conditions. The other half-leaf was macerated and tested for dye adsorption in the usual way, care being taken to record the halves by number for comparison of results. Five different groups of leaves, making a total of 64, were treated in this way, the leaves in any one group being in one flat and chilled simultaneously. The effect of the exposure to cold again was expressed in percentage of the half-leaf not injured, and the results on all of the different lots are summarized in table II. The results show a large number of leaves entirely

TABLE II

COMPARISON OF DYE ADSORPTION VALUES WITH FREEZING RESULTS FOR SEPARATE *BRYOPHYLLUM* LEAVES

SURVIVAL	DYE ADSORBED	MEAN	RANGE
%	%		
100	66–59–52–48–39–38–35–30–21	.....	.....
.....	11–5	36	66–5
95	95–74–59–44–29–25–5	47	95–5
90	56–54–52–43–41–31–26–14–10	36	56–10
85	60–10	35	60–10
75	50–	50	.....
70	53–	53	.....
50	44–	44	.....
40	15–	15	.....
35	34–	34	.....
25	40–36–16	30	40–16
15	37–34–	35	37–34
10	55–20	37	55–20
5	44–25	34	44–25
0	70–61–59–59–56–46–41–40–40	.....	.....
.....	39–34–34–31–28–26–24–13–5	.....	.....
.....	5–4–4	34	70–4

uninjured or slightly injured, a large number totally killed, and a few scattering ones with intermediate injury between. Also the large variation in the range of dye-adsorption values for the large groups of survival percentages is again encountered, as in the results for whole plants, and the mean values show no greater agreement to hardiness. The conclusion to be drawn from the experiments with *Bryophyllum* is that the dye-adsorption test is not effective in predicting hardiness in advance of freezing under the conditions of these experiments.

EXPERIMENTS WITH CABBAGE

This type of experiment was extended to cabbage, but, because the cabbage leaf will wilt more readily if detached from the plant for any length

of time, the half-leaves were left attached to the plant. The plants were quite large and after sampling, the whole plant, together with others in the group, were exposed to a temperature of – 5° C. for 15 hours. Two different groups of plants were tested, making a total of 40 plants, and the results appear in table III. No greater agreement of the dye-adsorption results

TABLE III

COMPARISON OF DYE ADSORPTION VALUES WITH FREEZING RESULTS FOR SINGLE CABBAGE LEAVES

SURVIVAL	DYE ADSORBED	MEAN	RANGE
%	%		
85	57–	57	.....
75	63–47	55	63–47
60	54–	54	.....
50	50–	50	.....
35	55–52–	53	53–52
30	51–50–49–48	49	51–48
25	47–	47	.....
20	57–54–46–40–34	46	57–34
15	67–56	61	67–56
10	61–52–43–42–38	47	61–38
5	45–	45	.....
0	69–58–56–56–55–55–52–	.....	.....
.....	50–44–42–41–41–40–39	49	69–39

with hardiness is evident here than with *Bryophyllum*.

Since these results indicate that the dye-adsorption test is not accurate enough to predict hardiness of small units of a plant such as a leaf, a further series of experiments was carried out to determine if it can predict

TABLE IV

COMPARISON OF DYE ADSORPTION VALUES ON COMPOSITE SAMPLES WITH FREEZING RESULTS FOR GROUPS OF CABBAGE PLANTS

GROUP NO. AND CONDITION OF PLANTS	LOT	NO. OF PLANTS PER LOT	TREATMENT	TISSUE SURVIVING	DYE ADSORBED
				%	%
1	A	10	Hardened 7 days at +5° C.	73	40
Good-sized, vigorous plants	B	10	Hardened 5 days at +1° C.	70	44
	C	10	Unhardened	38	35
2	A	12	Hardened 4 days at +5° C	71	56
Old plants	B	12	Unhardened	77	63
3	A	8	Hardened 6 days at +5° C.	25	64
Very old plants.....	B	8	Unhardened	20	63
4	A	25	Hardened 6 days at +5° C.	85	67
Young plants	B	25	Unhardened	65	63

with any accuracy the average performance of a large group of plants. Several different groups of cabbage plants of various ages were tested at different times. In each case half of the group was hardened by exposure to a low temperature slightly above 0° C. nightly for a few days, in an attempt to secure differences in cold reaction for comparison. Before being exposed to the lethal temperature of −5° C. for 15 hours each group was sampled for a dye-adsorption test by taking one fairly representative leaf from each plant. These were then macerated together and composite samples taken. A summary of the results appears in table IV. Here the results are not as conclusive as could be desired, especially in the second and third groups of old plants where the differences for the values of both tests are slight, but at least the hardier group of plants always gives the greater adsorption. It should be noted that hardening does not always produce a hardier lot of plants, as is shown in group 2.

Discussion

The literature on various tests and measurements of plant hardiness is very extensive and has been reviewed at great length in several recent papers. Therefore, none of it will be touched on here save to agree with MARTIN (**3**) and MAXIMOV (**4**): that none of the methods so far developed can be used with certainty to determine hardiness in a practical way as a substitute for a natural or artificial freezing test. Furthermore, it should be pointed out that the accuracy of many of these methods is determined by comparison with the varietal standings on the basis of general field and observational experience which, from results reported here and in previous papers (**1, 2**), is frequently very unreliable.

Summary

1. The dye-adsorption test involving colorimetric measurement of colloid content of plant tissues had previously shown some promise as a measure of cold hardiness. Further work is here reported to determine its accuracy in predicting hardiness in advance of freezing.

2. Tests on individual plants and even on individual leaves of *Bryophyllum* and cabbage gave contradictory results in comparison to subsequent freezing effects on the same plants and leaves, and indicate that the reliability of the test in prediction of hardiness is doubtful. This is similar to the conclusion reached for about every other method for testing hardiness reported in the literature.

3. A somewhat limited effectiveness of the method is indicated for tests of average hardiness of a large group of plants.

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ENVIRONMENTAL CONDITIONS INFLUENCING THE DEVELOPMENT OF TOMATO POCKETS OR PUFFS[1]

ARTHUR C. FOSTER AND EVERETT C. TATMAN

The condition known as tomato "pockets" or "puffs" is a serious disease or abnormal condition of the fruit of this crop in the Atlantic and Gulf Coast States, and frequently in California. This abnormal condition of the fruit is more prevalent in the mid-winter and early spring crops grown in Florida, and especially in the early spring crops grown in Mississippi and Texas. Frequently 15 per cent. of the total crop is lost in Texas, and often individual growers will lose as much as 65 per cent. of their crop. The malady is also of frequent occurrence on tomato crops grown in greenhouses in the north.

The literature relating directly to tomato "pockets" is limited and almost entirely of an empirical nature. However, there is an abundance of literature which relates indirectly to the phenomenon that contains considerable carefully collected experimental data. As early as 1892, MUNSON (3) observed that adequate pollen distribution was necessary for ovule fertilization and normal fruit development. WHITE (10) observed that insufficient pollen induced seedless tomato fruit with empty locules, which resulted in fruits of the typical pockets type. RADSPINNER (4) and SMITH (7), working in Oklahoma on the relation of environmental factors to anthesis and blossom-drop of tomatoes, came to the conclusion that hot dry winds and low soil moisture favor unusual elongation of styles which resulted in sterility and blossom-drop. SMITH and COCHRAN (8) reported that temperature had a marked effect on germination of pollen and rate of pollen tube growth. HOWLETT (2) reported that carbohydrate deficiency resulted in the suppression of the male organs and pollen sterility, while nitrogen deficiency did not affect microsporogenesis and the development of the male gametophyte.

The literature relating more directly to tomato pockets is rather fragmentary. SANDO (5) tried to determine the influence of various fertilizer combinations on the development of tomato pockets without definite results. He also failed to find very marked chemical differences between pocketed and normal fruit. PRITCHARD[2] working in Florida from 1924 to 1928, made extensive varietal trials with tomatoes and found that Marvelosa produced less pocketing of fruit than other varieties grown. SKINNER and RUPRECHT (6), working in Florida, reported that unfertilized tomatoes developed 80 per cent. of pocketed fruit, and that fertilized plants developed only 30 per

[1] See abstract under same title in Science, n.s. 86: 21–22. 1937.

[2] Preliminary report of experimental work on tomato disease. Bureau of Plant Industry, Reports of Progress, 1924 to 1928 inclusive. Unpublished data.

cent. of pocketed fruit. TRAUB, HOTCHKISS, and JOHNSON (9), working in Texas, gave the first detailed description of the malady and established a tentative classification of the different types. They also suggested the use of the term "pockets" in preference to the colloquial term "puffs." FRIEND (1), working in Texas, carried out extensive variety and fertilizer trials with tomatoes and came to the general conclusion that pocketing was not materially affected by the use of fertilizer, but that less loss occurred on plots receiving 600 pounds of superphosphate per acre. He also noted that, while all varieties tested were susceptible to pocketing, some appeared to be more susceptible than others. YARNELL (11), working in Texas, reported that pocketing was often the result of genetic factors affecting fruit development.

The present experiments were conducted in the greenhouse at Arlington Experiment Farm near Rosslyn, Va., during 1931 to 1935. The methods of procedure were rather unique. The plants were grown in a good quality of greenhouse soil made up of clay loam from Arlington Farm, composted with manure and muck, to which sand was later added. Before use, all soil was passed through a ¼-inch mesh wire screen to insure uniformity. This soil had a water-holding capacity of 65 per cent. of its dry weight, and the water-holding capacity remained uniform over a period of 5 years' experimental work.

The plants were grown in a wide range of approximate soil moisture levels, varying from 39 to 85 per cent. of the water-holding capacity of the soil. The soil moisture series were set up by first determining the original amount of water in the soil, and then adding sufficient water to bring the water content up to the required percentage. The soil moisture was maintained at approximately the desired level by frequent (4 to 6 times daily) additions of measured amounts of water, the required amounts being determined by weighing the cultures on solution balances of 40-kgm. capacity. All plants were grown in 3-gallon glazed crocks holding 12 kgm. of soil.

Fertilizer containing varying amounts of nitrate of soda, superphosphate, and potassium sulphate, at the rate of 2 tons per acre, was incorporated into the soil as it was placed in the crocks. During the 5-year experimental period, fertilizers with widely varying analyses, in the order of N, P, and K, respectively, were used: 0–12–12, 2–2–12, 2–12–2, 8–8–8, 12–0–12, 12–2–2, 12–12–0, and check or no added fertilizer, were used during 1931 and 1932; and two series of crops with these analyses were grown for two short and two long day periods. During 1933, other analyses were used: 0–0–12, 0–6–6, 0–12–0, 6–0–6, 6–6–0, 12–0–0, and check. Another crop was grown during midsummer of 1933 with fertilizer analyses varied and lime added, to obtain varying pH values of the soil from 4.5 to 8.5. In this experimental set-up, the source of nitrogen was varied so as to increase the alkalinity with increasing amounts of nitrate of soda with added lime; and

sulphate of ammonia was used to increase the acidity of the soil. The analyses used in this experiment were: check, 2–6–8, 4–6–6, 6–6–4, 8–6–2, and 10–4–2.

Either 5 or 6 crocks of each fertilizer treatment were placed in each of the 3, 4, or 5 soil moisture series, and these were grown under as uniform temperature as could be maintained in a greenhouse. A crop was matured during December or January for the short daylight period effect, and a duplicate was grown to mature during the middle of summer for the long daylight period effect.

During 1933 to 1935, the fertilizer analyses used were reduced to three: 12–0–12, 12–6–12, 6–12–6, and check or no added fertilizer.

In these experiments, 5 crocks of each fertilizer treatment were placed in each of the three soil moisture series, and these were replicated in each of the three different temperature units of the greenhouse, maintained at approximately 65°, 70°, and 75° F. This gave for study, 5 replications of each at the 36 points of observation, a total of 180 plants under approximately controlled environmental conditions.

The Marglobe variety of tomato was used throughout the experimental period of 5 years. The date of sowing the seed for the short and long day crops varied, but the seedling plants were transplanted to the 3-gallon crocks when about 3 weeks old, and allowed to become established. The plants were staked with heavy wire stakes and later, with the appearance of the first flower cluster, the wire stakes were replaced with 6-foot bamboo stakes. The plants were pruned to a single stem, tied to the stake, and eventually topped after the appearance of the sixth flower cluster. During the growth period, the moisture content of the soil was kept approximately constant by the method already mentioned. A careful record was kept of the total amount of water consumed by each plant daily for the duration of the various experiments.

The experimental method as outlined would indicate that the plants were grown under approximately uniform controlled environment, and this was true for the majority of the cultures. However, to determine the influence or effect of sudden changes in environment, selected plants from the different soil moisture and temperature cultures were changed to other environments and effects observed.

During the 5 years' intensive study of the environmental factors, namely, soil moisture, relative proportion of mineral nutrients, temperature, and length of day period, which influence the development of tomato pockets, a large amount of data has accumulated. These data represent a study of each individual plant, as to its daily and total transpiration rate and water requirement, and also a study of the effects of sudden increases or decreases in soil moisture. The effects of sudden changes in temperature and in evap-

oration capacity of the air have also been studied. The detailed data are too extensive to present in tabular form in a résumé, so a summary of the major results are given.

The most important conditions within the plant and the associated environmental factors that appeared to contribute to the pocketing of tomato fruits in these studies may be classified in three groups, and are as follows:

(1) *No fertilization of ovules or typical parthenocarpy.* It should be recognized that the culture of tomatoes in the greenhouse under varying environmental conditions offers ideal conditions for producing pocketing of the fruit. Under ordinary circumstances pollen distribution is poor but this was largely overcome by frequent agitation of the plants, by handling them in weighing to correct for water loss, in tagging the blossoms as they opened, in the tieing of the plants to the stakes, and in the taking of notes. In addition, the most prominent factors that contribute to sterility and parthenocarpy may be briefly noted: (a) abnormally long styles, a result of high temperatures; (b) slow germination of pollen, a result of low temperature; (c) slow growth rate of pollen tube, due to low temperature; (d) pollen abortion, a result of low carbohydrate reserve, caused by high nitrogen, high soil moisture, high temperature, and short daylight period; (e) ovule or embryo abortion, due to low nitrogen reserve, a result of low nitrogen, high soil moisture, high temperature and short daylight period. In these studies the many factors enumerated contributed strikingly to the development of pocketed fruit, especially in the high and low temperature greenhouse units when large amounts of nitrogen were applied.

(2) *Ovule or embryo abortion after normal fertilization.* The saturation or supersaturation of the soil appeared to cause marked changes in the normal metabolic, respiratory, and transpiration activities of the plant, which, in turn, resulted in ovule or embryo abortion. Also, excessive drought accompanied by high transpiration, a condition that apparently results in endoxerosis, causes marked apparent changes in normal metabolic, respiratory, and transpiration activities, and results in ovule and embryo abortion and the development of pockets.

(3) *Necrosis of vascular and placental tissue after fruit growth is well developed.* During any period of the growth of the plant, the saturation or supersaturation of the soil apparently stops almost entirely all transpiration, causes marked changes in color, the plants becoming chlorotic, and also otherwise interferes with the normal metabolic activities. These conditions apparently contribute largely to the appearance of necrosis of the vascular and placental tissue, which, in turn, leads to the development of pockets of the fruit of any age. Low soil moisture, accompanied by high transpiration, results in endoxerosis of the vascular and placental tissue. Sudden changes from optimum or high soil moisture to low soil moisture, accompanied by

excessive transpiration, appeared to be the most drastic treatment of all that favored the development of pockets.

Samples for chemical analyses, collected from the stem, bottom part of the plant, middle part of the plant, top part of the plant, and of fruit of different ages show marked differences in carbohydrate and nitrogen content. Significant correlations are to be expected with reference to the different plant parts and the appearance of pockets. The analyses of fruits from various treatments differ little when collected at the same stage of maturity, with the exception of those grown in the low temperature house. They are almost entirely vegetative in nature, and have a higher percentage of total solids, reducing sugars, and starch.

In these studies we are dealing with an environmental complex of many factors, as, for instance, soil moisture, soil nutrition, air temperature, light duration, and the interrelation and interaction of these component factors, any one of which may become a limiting factor to normal plant growth, and metabolic and respiratory activity, and thereby interfere with the normal development of tomato fruits. There is considerable suggested evidence that large amounts of superphosphate and only moderate amounts of nitrogen in the fertilizer reduce pockets by giving a nutritional balance conducive to more nearly normal seed development. The factors that can be observed and measured appear, when adverse, to bring about a general disturbance of the metabolism of the plant, causing a condition of suboxidation or endoxerosis, to affect the CO_2 and O_2 exchange, which, in turn, apparently leads to a visible necrosis of the vascular and placental tissue, thereby affecting ovule and embryo and placental development and normal fruit growth.

UNITED STATES DEPARTMENT OF AGRICULTURE
HORTICULTURAL FIELD STATION
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PHOTOPERIODIC STIMULATION OF GROWTH BY ARTIFICIAL LIGHT AS A CAUSE OF WINTER KILLING

PAUL J. KRAMER

(WITH ONE FIGURE)

The results of several recent investigations indicate that length of day or photoperiod is an important factor in determining the length of growing season in certain species of woody plants. The short days of late summer and autumn stop stem elongation and the production of new leaves in a number of species long before temperatures are low enough to check growth. This has been strikingly demonstrated by a hedge of *Abelia grandiflora* growing on the Duke University campus. *Abelia* is ordinarily quite hardy, but it has been observed that the plants in this hedge near the electric lights winter-kill badly, while those midway between the lights are never injured. A study was therefore made to determine the cause of this difference.

The entire hedge was trimmed September 25 and at that time it was quite uniform in appearance. By October 20, when the first light frosts occurred, the plants on each side of the lights for a distance of about five yards were distinctly different in appearance from those farther away. They bore numerous new shoots which were soft and succulent, and covered with pale, green leaves which contrasted strongly with the short, woody shoots, and bronzed, dark-green color of the leaves farther from the light. Difference in color and amount of growth was so marked that it could be seen at a distance of over 200 yards.

The number of new shoots produced since pruning was counted in a strip one foot wide at yard intervals from the lights. The results of 14 such counts were averaged and are shown in figure 1. It will be observed that, beginning five yards from the light, the number of new shoots decreased rapidly with increasing distance up to about 15 yards, where the number became constant. It was also observed that the plants near the lights continued to flower later in the season than the plants at a distance.

Late in November, after several hard freezes had occurred, counts were made of the number of frosted shoots at yard intervals from the lights. It was found that the tips of all the growing, succulent shoots had been killed back two to six inches. The averages of ten such counts are shown in figure 1. It will be observed that the greatest number of dead shoots occurred at three yards from the lights, which is also the point of greatest light intensity, and that no dead shoots were found at a distance of nine or more yards from the lights.

The light intensity amounted to only about two foot-candles at the brightest point, so it could not be measured satisfactorily with an ordinary

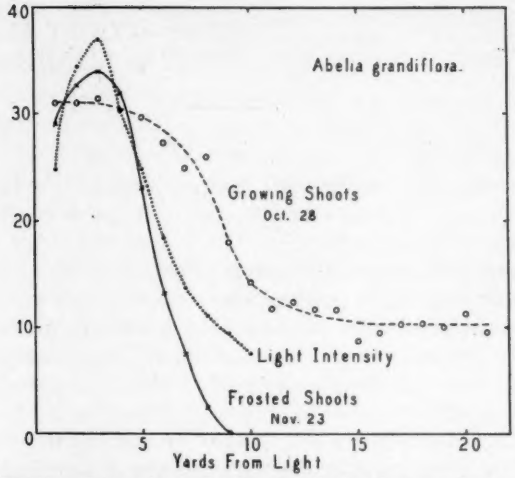

FIG. 1. Number of new shoots developed following last pruning; number of frosted shoots and light intensity measured at yard intervals from lights.

illumination meter calibrated in foot-candles. It was therefore measured at yard intervals from the lights by means of two photronic cells connected in parallel to a microammeter. The curve for light intensity represents the average of five determinations and is plotted in microamperes × 10. It will be noted that the number of new shoots produced after the last pruning, and the number of frosted shoots, are closely correlated with light intensity up to about ten yards from the lights. The most marked effects occur in the first five yards, nearly twice as many shoots being killed at five yards as at six yards. Since the maximum intensity was only two foot-candles, a light intensity of less than one foot-candle will produce a photoperiodic reaction in *Abelia*. The entire hedge is exposed to weak illumination from the lights, which burn all night; but at a distance greater than ten yards from the light the intensity is too low to affect growth.

Early in September, two dozen vigorous *Abelia* plants were removed from the nursery, potted, and placed outside the greenhouse. Half of them were exposed to normal length of day while the other half received daylight supplemented with sufficient electric light to give a photoperiod of about 14.5 hours, which equals the longest days of midsummer. By early October, only two of the normal-day plants had produced new shoots, while all of the long-day plants bore long, succulent shoots with light-green leaves. The new growth was killed back by November freezes, while the woody shoots and bronzed, dark-green leaves of the normal-day plants were entirely uninjured. It appears that a photoperiod equal to that of midsummer is just as effective

in prolonging the growing season as the continuous light to which the hedge is exposed.

Black locust (*Robinia pseudoacacia* L.) seedlings exposed to long and normal photoperiods reacted in the same manner as *Abelia*. The normal-day plants ceased growth in September while the long-day plants continued rapid growth until killed by freezing weather in November. The normal-day plants were entirely uninjured by these freezes.

Discussion

The results of these experiments clearly indicate that winter killing resulted from the failure of the *Abelia* to cease growth and become hardened before freezing weather occurred. The failure to cease growth can definitely be attributed to the photoperiodic stimulation of electric light. It is evidently unwise to plant *Abelia* too near bright lights. The same conclusions apply to black locust.

These results are in accord with those of certain Russian workers who have stressed the importance of photoperiodic reaction in determining the degree of cold resistance of woody plants. BOGDANOV (**1**) and MOSHKOV (**3**, **4**) found that certain southern species transferred to the long days of northern Russia continued to grow in the autumn until killed by frost. If the photoperiod was artificially shortened they ceased growth before freezing weather occurred, and survived the winter without injury. As the writer (**2**) has reported, not all trees are sensitive to a long photoperiod, and it is unlikely that all shrubs are sensitive. It is believed, however, that many instances of winter killing of isolated trees and shrubs result from proximity to electric lights causing growth to continue too late in the autumn. Several such occurrences have already been reported to the writer, and it is hoped that other investigators will search for similar examples. Information of this nature will enable those in charge of planting operations to avoid disappointments by keeping light sensitive plants away from bright lights.

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BRIEF PAPERS

DEVELOPMENT OF CAROTENOID PIGMENTS WITHOUT THE AID OF LIGHT

Xanthophyll and carotene are usually associated in plants and are present before the formation of chlorophyll. Their formulae suggest genetic relationship. Xanthophyll can be reduced to carotene and the transformation is reversible. Together they may represent a respiratory mechanism similar to the haemoglobin-oxyhaemoglobin complex in animal forms.

No chlorophyll develops in seedlings not exposed to light as was first recorded by JOANNES RAJUS in 1693. BONNET called such plants "etiollées" and the condition is now known as etiolation.

ARNAUD in 1889 first suggested that etiolated leaves contain carotene. The carotenoids are in leucoplastids which are generally assumed to be identical with the chloroplastids that contain the chlorophyll of normal plants. KRAUS first studied the alcoholic extract of etiolated leaves spectroscopically and concluded that the yellow pigments and the chlorophyll of normal plants are genetically related. PRINGSHEIM made similar studies in 1874 and concluded that a special pigment was present which he called "etiolin." This substance seems to be identical with protochlorophyll.

The results of experiments by the writer and by others before him, prove that chlorophyll forms within less than 30 seconds after etiolated plants are exposed to light. Protochlorophyll is probably the precursor.

According to observations of the writer the yellow color of etiolated sunflower seedlings is limited almost entirely to the cotyledons. The stems are white except for a small zone directly below the cotyledons. Upon exposure to light, this zone increases downward, the yellow extending beyond the green when chlorophyll forms. In time, however, the yellow and green colors become confused. It appears that some material is translocated in the stem which is converted into pigment in the plastid by the metabolic processes of the protoplasm.

The writer's experiments further show that the carotenoids are formed in sunflower seedlings without the aid of light. Some seeds contain carotenoid pigment, but attempts to extract them from sunflower seeds failed. Even if it is assumed that an initial amount was present, careful studies by the writer proved that the pigment forms in the absence of light. The greatest rate of development was between the fourth and seventh days of sprouting. The rate of increment in that time was much greater for carotene than for xanthophyll, and the decline from the seventh to the fourteenth day was much greater for the carotene. The amount of carotene developed, in comparison with the amount of xanthophyll, was always small.

The xanthophyll: carotene ratio increased during the period from the fourth to the seventh day, from 9 to 12, and during the period from the seventh day to the fourteenth day, from 13 to 20.

It may be concluded that the origin and the function of carotene and xanthophyll are probably related. The material stored in the seed probably is the source of the necessary material and energy required for the formation of the carotenoid pigments.

Carotene appears to have a stimulating effect on the production of roots, which suggests that carotene may be a precursor of auxin.—WILLIAM A. BECK, *Institutum Divi Thomae, Graduate School of Scientific Research, Cincinnati, Ohio.*

NOTES

Annual Election.—The annual election of the American Society of Plant Physiologists has resulted in the choice of the following officers for 1937–1938: President, Dr. OTIS F. CURTIS, Cornell University; vice president, Dr. WALTER F. LOEHWING, The State University of Iowa; secretary-treasurer, Dr. F. P. CULLINAN, United States Department of Agriculture, Beltsville, Maryland; elected member of the executive committee, Dr. R. B. HARVEY, University of Minnesota (term to expire in 1940); elected member of the editorial committee, Dr. H. R. KRAYBILL, Purdue University (term to expire in 1940).

Two vacancies have been filled by executive committee action. Dr. BURTON E. LIVINGSTON, The Johns Hopkins University, has been appointed to the executive committee for one year, vice the election of the retiring president to a three year term by ballot. To succeed Professor FRANCIS E. LLOYD, McGill University, who after twelve years of faithful service has resigned from the editorial committee, Dr. G. W. SCARTH, McGill University has been appointed for a five-year term.

Denver Meeting.—The meeting of plant physiologists in Denver was arranged under the joint auspices of the American Society of Plant Physiologists, the Western Section of the society, and the Physiological Section of the Botanical Society of America. The meeting opened on June 22 with a symposium on chlorosis and some of the minor elements. The Western Society for Soil Science joined in the meeting, and an attendance of over 100 attested the wide interest in this subject. The afternoon session of June 22 was devoted to a symposium on plant hormones under the leadership of Dr. F. WENT, California Institute of Technology. This symposium was exceptionally well arranged, and presented the developmental history of hormone research, supported by current investigations. The discussion was continued during the evening at a dinner for plant hormone physiologists at the Cosmopolitan Hotel. The dinner was attended by more than 40 interested scientists. Round table discussion was punctuated or accompanied by brief sketches of work in progress, and by a free discussion of controversial points by men having different concepts and points of view. The deep interest shown in this program indicates that scientists really enjoy the stimulus of such meetings, and should probably provide for skilfully developed round table discussions more frequently.

The morning of June 23 was occupied with the presentation of numerous short papers, and the afternoon was spent with section G, A.A.A.S., which was celebrating its 100th meeting. June 24 was featured by a symposium on drought resistance in plants, with Dr. A. H. HENDRICKSON as chair-

man. A dinner and smoker for plant physiologists on Thursday evening was attended by about 80 individuals. The toastmaster, Dr. J. P. BENNETT, University of California, announced the results of the annual election, and also the election of two new patrons of the society, Dr. BURTON E. LIVINGSTON, The Johns Hopkins University, and Dr. ALEXANDER P. ANDERSON, Tower View Laboratory, Red Wing, Minnesota. He then called upon president R. B. HARVEY, who responded with an address on the processing of citrus fruits, and upon Dr. GEORGE R. HILL, JR., who responded with an address on the control of smelter gas injury to vegetation.

Friday morning was again given over to a program of numerous short reports, and the afternoon to field trips; however, heavy rain dampened the enthusiasm for mountain climbing and picnicking.

The meeting as a whole reflects great credit upon the officers of the several organizations cooperating in its arrangement. It was as well attended, as successful, and as full of interest as the usual meetings in December.

New England Section.—The annual meeting of the New England Section was held at the Rhode Island State College, Kingston, Friday and Saturday, May 14–15, 1937. An interesting program of short papers was arranged for Friday afternoon. This covered such problems as auto-irrigation of cultures, pH and soluble iron from ferric phosphate, embryo development of ash seeds previous to germination, influence of Bordeaux spray on tomatoes, calibration of recording illuminometers, sampling of light under forest canopies, and the use of cellulose films for supplementary insulation of plants under glass.

Friday evening was given over to the annual dinner, with an address of welcome from President BRESSLER, and an illustrated lecture on controversial conservation by HENRY E. CHILDS.

Saturday morning was planned to give opportunity to observe the field plots, greenhouses, and laboratories of the Rhode Island Agricultural Experiment Station, and the State College. Another session for presentation of papers followed the tour of the experimental fields where so many interesting discoveries have been made. In the midst of this program the annual business meeting was held as an intermission period. The officers of the New England section elected for 1937–1938 are Dr. F. H. STEINMETZ, University of Maine, chairman; Dr. H. B. VICKERY, Connecticut Agricultural Experiment Station, vice chairman; and Dr. L. H. JONES, Massachusetts Agricultural Experiment Station, was reelected secretary-treasurer. The fourth annual meeting of the section will be held at Orono, Maine, in May, 1938, under the auspices of the University of Maine.

Saturday afternoon was devoted to a teachers' forum which attracted much attention. It was a seminar on the teaching of plant physiology, deal-

ing with the logical objectives and methods of approach in teaching plant physiology. The committee members in charge of this new departure were Dr. C. J. LYON, Dartmouth College, chairman, Dr. DOROTHY DAY, Smith College, Dr. CARL G. DEUBER, Yale University, and Dr. F. H. STEINMETZ, University of Maine. This meeting was so significant and so valuable that a special report from the chairman of the forum committee will be published in the October number of PLANT PHYSIOLOGY. It is a sign of healthy progress that deep interest is being shown in the different pedagogical techniques demanded in handling plant physiology in the agricultural colleges, and in liberal arts colleges. It is hoped that future seminars along similar lines may bring the problems of effective teaching of this subject to the forefront of interest among plant physiologists throughout the United States.

Corporate Seal.—The American Society of Plant Physiologists was incorporated as a non-profit organization under the laws of the District of Columbia, July 5, 1932. Under the revenue laws relating to exemption of non-profit organizations from income taxes, it was necessary to have a corporate seal. During the last year the secretary has given attention to the development of an appropriate design, which was submitted to the executive committee at the Denver meeting for approval. The executive committee authorized the adoption of a corporate seal, approved with slight change the design submitted, and authorized the secretary to have it made up immediately. We hope that a reproduction of the design may be printed as soon as it is available.

Legislative Committee.—The executive committee authorized the appointment of a legislative committee at the Denver meeting, the vice president of the society to be chairman of the committee. Dr. C. O. APPLEMAN, University of Maryland, was named for a two-year term on the committee, and Dr. CHARLES B. LIPMAN, University of California, to a three-year term. Dr. W. F. LOEHWING, vice president, will be chairman during 1937–1938.

Hans Molisch.—It has seemed appropriate to honor those who have been elected as corresponding members of the American Society of Plant Physiologists by some form of public recognition of their service. It is therefore with great pleasure that we are able to present in this number of PLANT PHYSIOLOGY a portrait of HANS MOLISCH, accompanied by a paper from L. LINSBAUER on *Fifty years of Plant Physiology in Austria.*

HANS MOLISCH was born at Brünn (Mähren) on July 12, 1856, and celebrated his eightieth birthday anniversary last year. His parents were JOHANN and FRANZISKA (MATZA) MOLISCH. He was educated at the K. K. Deutschen Ober-Gymnasium at Brünn, and at the University of Vienna,

which institution he entered in 1885. On March 10, 1888, he was married to IDA KNOLLER, who is the mother of two children, Paul, born in 1889, and Fritz, born in 1894.

In 1889 HANS MOLISCH became ausserordentlicher Professor at the Technische Hochschule in Graz. Several years later he moved to Prag, where in 1894, he was appointed ordentlicher öffentlicher Professor in the German university and director of the Plant Physiological Institute. In 1909 he returned to Vienna with the same title. During 1922 to 1925 he was visiting professor at the Imperial University at Sendai, Japan, and travelled extensively in the Orient. Since 1925 he has been at the University of Vienna as emeritus professor of plant physiology. Throughout these years of his professional life he was very active in his investigations, and has published over 200 papers on plant physiology. He is also well known for his many books (about 20), some of which were standard textbooks used in the universities, while others served to increase popular interest in physiological experiments. He was elected a corresponding member of the American Society of Plant Physiologists at St. Louis in 1935.

At the beginning of his eighty-second year, we are happy to extend to him the cordial greetings of American plant physiologists, who appreciate the great contributions he has made to our knowledge of plants during the last half-century, and who admire him as an indefatigable worker in this field of science.

Edward C. McCarty.—News of the sudden death of Dr. EDWARD C. MCCARTY at his home in Riverside, California, on December 31, 1936, at the age of 52 years, came as a severe shock to his many friends throughout the country. Dr. MCCARTY was born in 1884, and held degrees from the Jefferson School of Law (Doctor of Laws, 1912), from the University of Montana (B.S., 1916), and from the University of California (Ph.D., 1927). He was associate professor of plant physiology at the Colorado Agricultural College from 1920 to 1926, and during the last 10 years had been on the faculty of the Riverside Junior College. Here he combined in happy manner his aptitude as a teacher, his fondness for investigation, and his love of students. His research on range problems attracted attention of the U. S. Department of Agriculture and he was employed during the summer in the work of the Forestry Division. His work on grasses and their responses to grazing are particularly noteworthy. One of these contributions was published in PLANT PHYSIOLOGY **10**: 727–738. 1935.

Gifted with an unusually friendly personality, he was a wise counsellor and friend to all of his students and colleagues. His industry, his honesty, his loyalty to his associates and to his own ideals, his humility of spirit endeared him to all who knew him; and his departure represents an irreparable

loss to his family, his friends, his institution, and to his field of science. He was for several years a member of the American Society of Plant Physiologists.

The immediate cause of death was a heart attack. Funeral services were held on the morning of January 4, 1937, at the First Congregational Church in Riverside.

Annual Review of Biochemistry.—The sixth volume of the *Annual Review of Biochemistry* has just been published. It is not only a very creditable example of the art of book making; it is also a volume whose usefulness and value would be difficult to overstate. The reviews are carefully prepared. Everyone who has tried to review the progress of any given field must realize the difficulty of the task undertaken in this series; and it is remarkable that year after year the work is so ably done. This indicates a faithfulness and responsibility on the part of editors and reviewers that is worthy of great praise. No biochemist, no physiologist can afford to omit it from his list of indispensable reference works.

The 1937 volume contains 28 reviews, many of which are of interest to plant physiologists. Some of the most important of these are mentioned to give some idea of the scope of the review. The first paper is on permeability, by R. COLLANDER; others are on biological oxidations and reductions, by F. LIPMANN; enzymes, by K. LINDERSTRØM-LANG; the application of microchemistry to biochemical analysis, by P. L. KIRK; the chemistry of carbohydrates and glycosides, by W. N. HAWORTH and E. L. HIRST; the chemistry of the lipins, by E. KLENK and K. SCHWIRTH; the chemistry of the steroids, by R. SCHOENHEIMER and E. A. EVANS, JR.; the chemistry of the proteins and amino acids, by C. S. ADAIR; detoxication mechanisms, by A. J. QUICK; plant pigments, by J. H. C. SMITH; the alkaloids, by E. SPÄTH; photosynthesis, by R. EMERSON; mineral nutrition of plants, by F. G. GREGORY; organic acids of plants, by T. A. BENNETT-CLARK; and biochemistry of bacteria, by C. B. VAN NIEL.

The other reviews on metabolism, vitamins, hormones, etc., are mainly reviews of progress in the animal field. Thus a fair balance is maintained in the entire field of biochemistry. It represents an outstanding value among recent scientific works. An important valuable addition to volume VI is a subject index. This feature is so useful, and so necessary to rapid reference to the material that it should be included in each succeeding volume.

The editors, reviewers, and publishers deserve the success which has attended this series from its inception. Volume VI costs but $5.00, and may be ordered from Annual Review of Biochemistry, Ltd., Stanford University, California.

The Minor Elements.—A second edition of the *Bibliography of References to the Literature of the Minor Elements and their Relation to the Science of Plant Nutrition* has been issued by the Chilean Nitrate Educational Bureau Inc., 120 Broadway, New York. The original edition of 1935 was compiled by Prof. L. G. WILLIS, soil chemist at the North Carolina Agricultural Experiment Station. It contained about two thousand abstracts, and was so popular that this first edition was soon exhausted. The new edition contains 396 pages, and nearly a thousand new references and abstracts. It is a very valuable compilation and ought to be in the hands of every plant physiologist. Those who find references that are not included in the second edition are invited to send them to the Chilean Nitrate Educational Bureau. Those who wish to obtain a copy of the second edition should write to Mr. HERBERT C. BREWER, director of the Bureau. As the supply is already low, there is a possibility that some may have to be disappointed. They are supplied free of charge as long as they last.

Monograph on Growth.—Under the title *Studies on Wheat Grown under Constant Conditions*, the Food Research Institute, Stanford University, California, has published a valuable monograph on the growth of the wheat plant. The author of the monograph, Dr. H. L. VAN DE SANDE-BAKHUYZEN, will be remembered for his papers published in PLANT PHYSIOLOGY in 1928. The material of those papers is incorporated in the monograph, with a large mass of new data. He was assisted in parts of the work by ELIZABETH P. GRIFFING, and Dr. CARL L. ALSBERG of the Food Research Institute. There are seven sections or parts to the monograph, with 36 chapters in all. The main divisions of the monograph bear titles as follows: Part I, the growth curve in annual plants; II, methods and materials; III, general growth features; IV, dry weight and moisture content of the various organs; V, dry weight and moisture of the standard plant; VI, nitrogen and carbon of the organs; and VII, nitrogen metabolism in relation to growth and development. In addition to numerous figures and tables, there is a bibliography with 233 citations, and a brief index. The entire work occupies 400 pages.

Few agricultural plants have been investigated as carefully as the wheat plant has been for the preparation of this monograph. The program of research was both extensive, and intensive. A very good beginning has been made toward the elucidation of the growth behavior of wheat. This monograph should stimulate studies of other important plants along similar lines. Plant physiologists and students of general growth problems will find it of great value. The price of the monograph is $4.00 per copy, and orders may be addressed to the Food Research Institute, Stanford University, California.

The Nature and Properties of Soils.—This well-known textbook by Dr. T. L. LYON and H. O. BUCKMAN of Cornell University has appeared in a third edition. The work has been thoroughly revised to keep it in line with the changing emphasis in the study of soils. Some changes have been made also in the organization of the material, so that there are now only 17 chapters, some of which are new. One of these, chapter IV, deals with colloidal clay and ionic exchange. All of the material is valuable to the plant physiologist, and the last 5 chapters present a valuable survey of the problems of the maintenance of soil fertility. These are: Liming the soil; the nitrogen economy of soils; fertilizers and fertilizer practice; farm-manure and green-manure; and the methods of fertility maintenace for mineral soils. The book has not been much increased in size, it maintains its readable style, is not too technical for average students, and should make friends of all who are engaged in the problems of soil science and plant science. The book may be ordered from Macmillan Co., at $3.50 per copy.

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